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MINERAL REQUIREMENTS FOR GROWTH OF *STAPHYLOCOCCUS PYOGENES*. EFFECT OF POTASSIUM IONS

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Received for publication April 11, 1956

In an earlier study of the growth requirements of *Staphylococcus pyogenes*, magnesium and calcium were found to be necessary (Shooter and Wyatt, 1955). Using broth which had been made deficient in calcium and magnesium by passage through an ion exchange resin, the need for the missing elements was gauged from the significant and reproducible restoration of growth that followed their return to the medium. More rigorous extraction of broth has resulted in the removal of larger amounts of potassium in addition to magnesium and calcium, and this paper records the growth of *Staph. pyogenes* in a medium initially deficient in these three elements, with particular reference to potassium.

MATERIALS AND METHODS

Cleaning of glassware

The previous procedure was followed (Shooter and Wyatt, 1955) with the exception that all glassware was immersed in potassium dichromate-sulphuric acid for 18 hr.

Reagents

Except that re-distilled HCl was used for adjusting the pH of the medium, there was no change in the reagents.

Medium

Twice as much resin was used as before. Each 3 l. of broth (1 per cent solution of Lab Lemco (Oxo Ltd.) with 1 per cent peptone (Evans) and 0·5 per cent glucose) after removal of phosphates and adjustment of pH to 7·3 was divided into four portions, and each portion run down a 1·1 cm. diam. Pyrex tube filled for a length of 140 cm. with IRC-50 (Na^+). The pH was finally adjusted to 7·3 with HCl. The broth contained 0·0075 mg. ions/l. K^+ as compared with 0·075 mg. ions/l. previously. Sodium rose from 87 to 97 mg. ions/l.

Potassium measurement and potassium uptake

Potassium was measured with an EEL flame photometer, solutions used for calibrating this instrument being made up in a solution of 100 mg. ions/l. Na^+.

Measurements of potassium uptake by *Staph. pyogenes* have previously been made by flame photometry of the bacteria re-suspended. This has been necessary as the amount of

K^+ removed by bacteria has been very small compared with the K^+ concentration of the medium (Davies, Folkes, Gale and Bigger, 1953; Cooper, 1955). A K^+-deficient medium enables the K^+ content of the supernatant medium to be determined directly by flame photometry before and after growth. The K^+ uptake is obtained by subtraction.

Inoculum

Most of the work was done with one strain ("Terry") of *Staph. pyogenes*, which we had used before. It was kept on agar slopes, fresh cultures being made monthly.

The inoculum was reduced. Plate counts showed that approximately 4500 bacteria were added to each tube of 10 ml. of medium.

For measurements of growth and potassium, broth was dispensed in 8 ml. amounts in 6 in. × $\frac{3}{4}$ in. (15·2 × 1·9 cm.) tubes, and made up to 10 ml. with reagents and water. After inoculation and incubation for 18 hr. bacteria were suspended by scraping with a glass rod and shaking, and half the contents of each tube was placed in a centrifuge tube. This was centrifuged for 20 min. at 1800 *g* and the supernatant for K^+ estimation pipetted into glass cups. Bacterial growth was estimated turbidimetrically with a Spekker absorptiometer, using H508 filters and 1 cm. cells, after the addition of 5 drops of 40 per cent formalin to the remaining half of the suspension in the culture tube.

There was no detectable contribution of K^+ to the medium by the inoculum or the reagents of Ca^{2+} and Mg^{2+}.

Dry weight

Samples of 10–20 mg. staphylococci in weighed centrifuge tubes were dried at 110° for 16 hr., cooled over P_2O_5 *in vacuo* and weighed. The samples were of 18 hr. growth in the control untreated broth. Measurements with the Spekker were referred to a calibration curve and converted to dry weight. It is realised that the dry weight/opacity relationship may not be constant for growth in deficient medium. Absorptiometer readings after 18 hr. growth varied between 0·530 and 0·800 with the strain of staphylococcus used. A reading of 0·650 for the strain used corresponded with a dry weight of 2·6 mg. per 10 ml.

Measurement of pH

The pH of the supernatant broth was measured, after the K^+ determinations, with a Marconi pH meter using glass and calomel electrodes. Trials showed that the pH was not altered by centrifugation.

RESULTS

After passage through the resin the broth did not support growth, and the addition of 0·8 mg. ions./l Mg^{2+} and 0·5 mg. ions/l. Ca^{2+} without K^+ resulted in less than 5 per cent of the control growth as compared with 70 per cent obtained with 0·08 mg. ions/l. $Mg.^{2+}$ and 0·05 mg. ions/l. Ca^{2+} added to the less rigorously treated medium (Shooter and Wyatt, 1955).

Effect of Potassium on Growth

The return of up to 10 mg. ions/l. K^+ alone to the treated medium gave no growth. Addition of up to 2 mg. ions/l. K^+ and 0·5 mg. ions/l. Ca^{2+} gave up to 6 per cent growth and addition of Mg^{2+} and K^+ gave up to 50 per cent growth with 0·8 mg. ions/l. Mg^{2+} and 2 mg. ions/l. K^+. Growth in the absence of Ca^{2+} was erratic, so the main experiments were conducted with additions of the three elements K^+, Ca^{2+} and Mg^{2+}.

Fig. 1 shows the results of returning K^+ to broth containing insufficient and sufficient amounts of Mg^{2+} and Ca^{2+}. In each case, with increasing K^+ there was a steady increase of growth to between 40–50 per cent of the control growth. At this point, which lay between 0·056 and 0·1 mg. ions/l. K^+, further growth in the

lower concentrations of Ca^{2+} and Mg^{2+} was prevented by a deficiency of Ca^{2+} and Mg^{2+}. However, when both were present in larger amounts, growth continued to rise to nearly 70 per cent with 0·5 mg. ions/1. K^+.

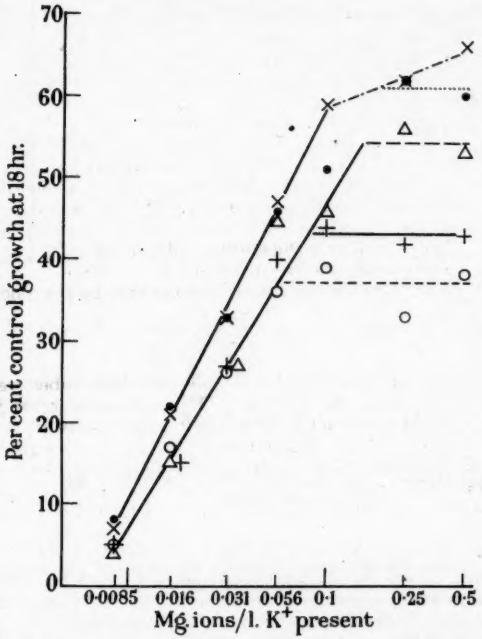

FIG. 1.—Effect on the growth of *Staph. pyogenes* at 18 hr. of the addition of K^+, Mg^{2+} and Ca^{2+}. (Broth batch L.8.)

○ 0·04 mg. ions/l. Mg^{2+} and 0·005 mg. ions/l. Ca^{2+}.
● 0·04 „ „ Mg^{2+} „ 0·05 „ „ Ca^{2+}.
+ 0·08 „ „ Mg^{2+} „ 0·005 „ „ Ca^{2+}.
× 0·2 „ „ Mg^{2+} „ 0·05 „ „ Ca^{2+}.
△ 0·8 „ „ Mg^{2+} „ 0·005 „ „ Ca^{2+}.

Fate of Potassium

Uptake of potassium by Staph. pyogenes

Potassium uptake, calculated by subtracting the residual K^+ in the medium after 18 hr. growth from the K^+ initially present, was determined in the presence of rising K^+ concentrations at the five different levels of Mg^{2+} and Ca^{2+} shown in Fig. 1. In each case K^+ uptake increased as growth increased with rising K^+ content of the medium, and while growth increased, the bulk of the K^+ was taken up by the organisms. At the stage at which K^+ ceased to be the limiting factor, and additional K^+ produced no further increase in growth, K^+ uptake also remained stationary and the residual K in the medium rose sharply.

There was the difference, however, that with inadequate Mg^{2+} and Ca^{2+} (0·08 and 0·005 mg. ions/1.) the residual K^+ remained at a low level while the K^+

uptake rose (Fig. 2*a*), whereas with sufficient Ca^{2+} the residual K^+ rose even although growth was dependent on K^+ (Fig. 2*b*). The K^+ uptake with tubes containing 0·04 mg. ions/l. Mg^{2+} and 0·005 mg. ions/l. Ca^{2+} was similar to that shown in Fig. 2*a*, and uptake with tubes containing 0·2 mg. ions/l. Mg^{2+} and 0·05 mg. ions/l. Ca^{2+} was similar to that shown in Fig. 2*b*.

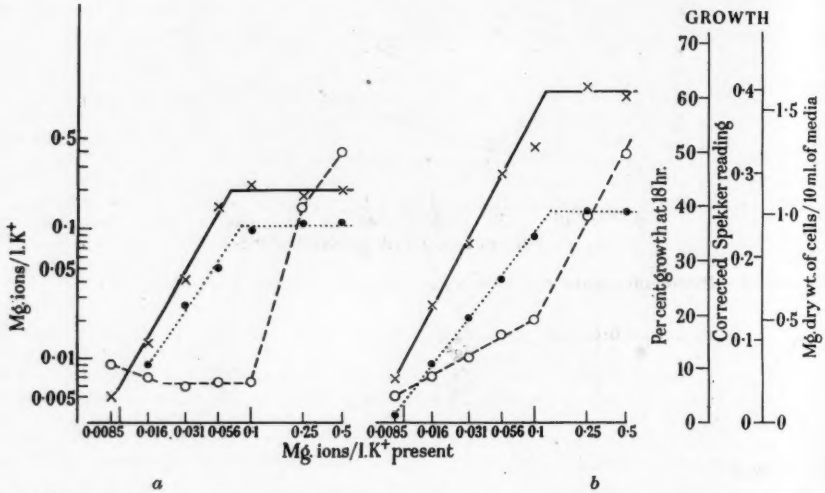

FIG. 2.—Potassium uptake by *Staph. pyogenes* in the presence of insufficient and sufficient amounts of Ca^{2+}.

2*a* 0·08 mg. ions/l. Mg^{2+} and 0·005 mg. ions/l. Ca^{2+}.
2*b* 0·04 „ „ Mg^{2+} „ 0·05 „ „ Ca^{2+}.
× growth.
○ residual K^+.
● K^+ taken up by bacteria.

The K^+ uptake with 0·8 mg. ions/l. Mg^{2+} and 0·005 mg. ions/l. Ca^{2+} differed from both these patterns. Up to an initial K^+ concentration of 0·031 mg. ions/l. uptake was nearly complete, but at greater K^+ concentrations the residue increased slowly.

Influence of calcium on the uptake of potassium by Staph. pyogenes.

The uptake of K^+ by *Staph. pyogenes* is shown in Fig. 3. The K^+ uptake per cell increases gradually up to 40-50 per cent control growth and then increases rapidly. In the series of tubes containing 0·005 mg. ions/l. Ca^{2+} the point of inflexion is at 50 μmoles K^+/100 mg. dry wt., and in the presence of 0·04 and 0·08 mg. ions/l. Mg^{2+} there is a great increase in the K^+ uptake with little increase in growth. In the series of tubes containing 0·05 mg. ions/l. Ca^{2+} the point of inflexion is at 40 μmoles K^+/100 mg. dry wt. and the increase after this is accompanied by an increase in growth. The tubes containing 0·005 mg. ions/l. Ca^{2+} and 0·8 mg. ions/l. Mg^{2+} show a K^+ uptake after the point of inflexion midway between the two main Ca^{2+} series.

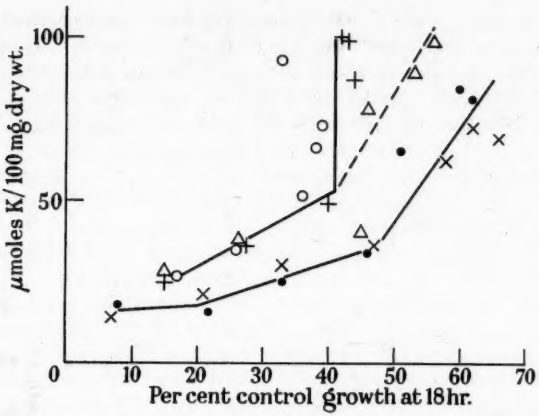

FIG. 3.—Potassium uptake by *Staph. pyogenes* in the presence of varying amounts of K^+, Mg^{2+} and Ca^{2+}.

○ 0·04 mg. ions/l. Mg^{2+} and 0·005 mg. ions/l. Ca^{2+}.
● 0·04 „ „ Mg^{2+} „ 0·05 „ „ Ca^{2+}.
+ 0·08 „ „ Mg^{2+} „ 0·005 „ „ Ca^{2+}.
× 0·2 „ „ Mg^{2+} „ 0·05 „ „ Ca^{2+}.
△ 0·8 „ „ Mg^{2+} „ 0·005 „ „ Ca^{2+}.

Change in pH

The pH of the control broth fell after 18 hr. from 7·3 to 5·0. In the treated medium there was no change at the lowest levels of growth and the lowest K^+ concentration. Thereafter the pH fell with increasing growth and increasing K^+ concentration (Fig. 4). Up to 30 per cent of growth all the combinations of Ca^{2+} and Mg^{2+} showed the same change in pH with equal growth. Beyond this point the two sets of cultures with sufficient Mg^{2+} but too little Ca^{2+} produced the least change, although the pH fell with increasing K^+ even when there was no increase in growth. In the other Mg^{2+} and Ca^{2+} combinations the pH continued to fall with increasing K^+ and increasing growth, although as shown in Fig. 4, the growth at each pH level was different for each series.

General characteristics of growth in deficient media

Cultures in media containing 0·04 and 0·08 mg. ions/1. Mg^{2+} together with 0·005 mg. ions/1. Ca^{2+} were abnormal in that the bulk of the fluid was clear, bacteria being aggregated at the bottom of the tube. Cultures containing 0·8 mg. ions/1. Mg^{2+} or 0·05 mg. ions/1. Ca^{2+} produced more uniform growth.

Growth of other strains of Staph. pyogenes

These results have been obtained with one strain (" Terry ") of *Staph. pyogenes*. The Table shows the results of growing " Terry " and three other strains in varying concentrations of K^+, Ca^{2+} and Mg^{2+}. All four strains were limited by K^+ and by Ca^{2+}, and three of the strains showed little extra growth with increase of K^+ above 0·1 mg. ions/1.

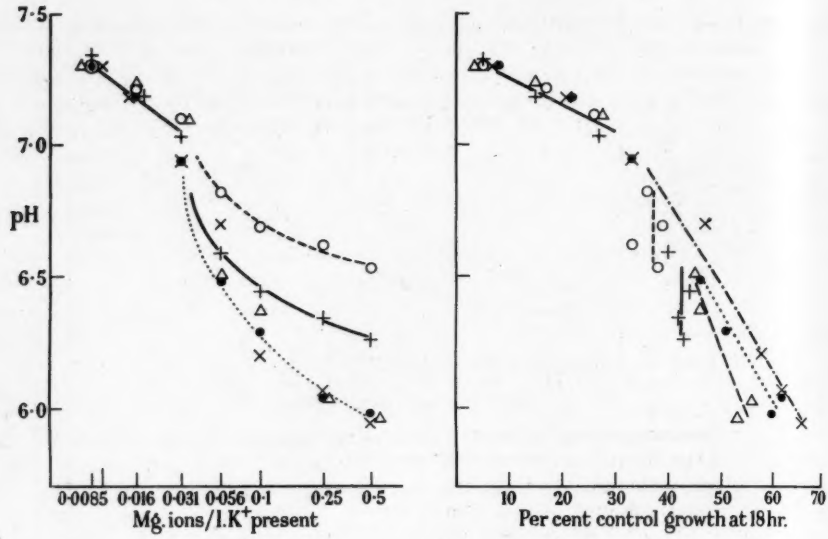

FIG. 4.—pH changes in cultures of *Staph. pyogenes* growing with varying amounts of K^+, Mg^{2+} and Ca^+.

○ 0·04 mg. ions/l. Mg^{2+} and 0·005 mg. ions/l. Ca^{2+}.
● 0·04 „ „ Mg^{2+} „ 0·05 „ „ Ca^{2+}.
+ 0·08 „ „ Mg^{2+} „ 0·005 „ „ Ca^{2+}.
× 0·2 „ „ Mg^{2+} „ 0·05 „ „ Ca^{2+}.
△ 0·8 „ „ Mg^{2+} „ 0·005 „ „ Ca^{2+}.

TABLE.—*Growth at 18 hr. of 4 Strains of* Staph. pyogenes *with Varying Concentrations of K^+, Mg^{2+} and Ca^{2+}. Results expressed as Spekker Readings (Broth batch L.7)*

Magnesium mg. ions/l. added.	Calcium mg. ions/l. added.	Potassium mg. ions/l. present.	Strains of *Staph. pyogenes.*			
			"Terry."	"Berkof."	"Clarke."	"Eldridge."
0·02	0·05	0·011	0·075	—	0·040	0·040
0·02	0·05	0·1	0·304	0·422	0·303	0·292
0·02	0·05	0·5	0·322	0·400	0·335	0·275
0·2	—	0·031	0·075	0·055	0·168	0·115
0·2	—	0·25	0·188	0·155	0·321	0·173
0·2	—	1	0·212	0·208	0·355	0·120
Control	—	—	0·650	0·530	0·720	0·800

Strains "Berkof" and "Clarke" were sensitive to penicillin and oxytetracycline. "Eldridge" was resistant to both antibiotics. "Terry" was resistant to penicillin and sensitive to oxytetracycline.

DISCUSSION

Potassium has been found to be necessary for the growth of *Clostridium welchii* (Shankar and Bard, 1952), lactic acid bacteria (MacLeod and Snell, 1947) and *Bacterium coli* (Friedman and Fox, 1954). The failure to obtain growth in the absence of potassium and the reproducible and significant increase in growth

which we have obtained on returning potassium to the depleted medium shows that potassium is also necessary for the growth of *Staph. pyogenes*.

Flame photometry has made it possible to determine the potassium uptake during growth. Within the range in which potassium limits growth, almost all the potassium in the medium is removed, and the content of the cell increases some six times. The residual figure of less than 0·01 mg. ions/l. may represent unavailable potassium or the minimum extra-cellular level required for growth.

The minor variations in potassium uptake shown in Fig. 2*a* and *b* may represent the partial interchangeability of calcium, magnesium and potassium. In the presence of insufficient magnesium and calcium nearly all the available potassium is taken up (Fig. 2*a*). With sufficient calcium rather less potassium is removed (Fig. 2*b*), and the results with excess magnesium but deficient calcium suggest that magnesium may be taking the place of calcium.

We have already suggested that one function for calcium is to take part in the formation of proteases which enable the staphylococcus to obtain amino acids from larger nitrogen residues. The results shown in Fig. 3 again demonstrate the necessity for calcium, in that with adequate calcium growth reaches approximately 60 per cent of the control growth, while with inadequate calcium less cell division may take place, although as much potassium is present per estimated milligram dry weight as at the higher growth ranges.

The fall in pH with rising potassium concentration is probably an indication of glucose fermentation, and in Fig. 4 the lowest pH is seen linked with maximum growth. Changes in pH are not associated with potassium uptake or necessarily with growth, and calcium is again involved (Fig. 4*b*).

SUMMARY

In order to determine the mineral requirements for growth of *Staph. pyogenes* a deficient medium has been made by passing nutrient broth through an ion exchange resin. The return of calcium, magnesium and potassium to this broth has resulted in a reproducible and significant increase in growth and has shown that potassium, in addition to calcium and magnesium, is an essential nutrient for *Staph. pyogenes*. Measurements have been made of the uptake of potassium by staphylococci and of the changes in pH which accompanied growth.

We would like to express our thanks to the Treasurer and Board of Governors of St. Bartholomew's Hospital for a grant which enabled this work to be carried out.

REFERENCES

Cooper, P. D.—(1955) *J. gen. Microbiol.*, **13**, 22.
Davies, R., Folkes, Joan P., Gale, E. F. and Bigger, L. C.—(1953) *Biochem. J.*, **54**, 430.
Friedman, S. and Fox, C. L.—(1954) *J. Bact.*, **68**, 186.
MacLeod, R. A. and Snell, E. E.—(1947) *J. biol. Chem.*, **170**, 351.
Shankar, K. and Bard, R. C.—(1952) *J. Bact.*, **63**, 279.
Shooter, R. A. and Wyatt, H. V.—(1955) *Brit. J. exp. Path.*, **36**, 341.

IN VITRO STUDIES ON HUMAN SYNOVIAL MEMBRANE. A METABOLIC COMPARISON OF NORMAL AND RHEUMATOID TISSUE

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IN order to assess the significance of *in vitro* findings of the action of anti-rheumatic agents on the rheumatoid synovial membrane, it is necessary to know to what extent the metabolism of the normal synovial membrane is affected by the disease process. Bywaters (1937) has shown that in man, cartilage and synovial membrane have the same type of metabolism as in the horse, and he also found no increase in glycolysis in a knee that showed marked rheumatoid changes.

In a previous paper (Page Thomas and Dingle, 1955) a comparison of the *in vitro* metabolic activity of rheumatoid synovial villi and membrane was made, with reference to oxygen uptake, glucose utilisation and lactate production. Under these conditions villi showed a greater metabolic activity. Since proliferation of the synovial villi is one of the earliest and most marked responses of the synovium to the disease stimulus, we felt it would be of interest to find whether any relationship existed between the metabolic state of the tissue and the local activity of the disease.

METHODS

"Normal" tissue was obtained at the time of operation from patients undergoing meniscectomy. These cases had a traumatic aetiology, and effusion had subsided before operation. There was usually some residual subsynovial engorgement of the capillaries on histological examination (Fig. 1).

Rheumatoid synovial tissue was similarly obtained from patients undergoing synovectomy or arthrodesis of the knee. For the purpose of this study, the rheumatoid synovial tissue was divided into three classes, depending on its naked-eye appearance.

Many tissues showed numerous free villous processes. Fig. 2 shows the characteristic florid appearance of tissue in this class, which we designated "villous".

Some villous tissues were less engorged, and numbers of these villi were frequently attached together at their tips, resulting in a general appearance more fibrotic in character than the previous class. We have classified this type of villous tissue as "fibrotic". The typical histological picture of this type is shown in Fig. 3.

Fig. 4 illustrates the third class of rheumatoid tissue, classified as "membrane", which was obtained from the non-villous areas of the synovium. All rheumatoid synovia used in this study were shown to have histological changes characteristic of the disease.

The tissues were prepared for *in vitro* studies as previously described (Page Thomas and Dingle, 1955). For aerobic experiments 100 per cent oxygen was used as the gas phase in the flasks, and for anaerobic work nitrogen (previously freed from traces of oxygen by yellow phosphorus) was employed. The appropriate gas was introduced into the chilled flasks by the evacuation procedure described by Umbreit, Burris and Stauffer (1951). All flasks had a 10-min. equilibration period at 37° before manometric measurements were begun. Unless otherwise mentioned all substrates used were at a final concentration of 10 mM. After incubation the separation of the tissue and the chemical estimations were carried out as

previously described. Aerobic glycolysis $\frac{Q_{O_2}}{LA}$ and anaerobic glycolysis $\frac{Q_{N_2}}{LA}$ were calculated from the chemical estimation of lactic acid. The results were expressed on a tissue nitrogen basis, denoted by $Q(N)$, but for comparison with the figures of other authors, the nitrogen/dry weight ratio was determined on a number of tissues. This ratio was found to be relatively constant, the mean figure being 1 mg. nitrogen/10 mg. dry weight.

RESULTS

These results, obtained over a period of 2 years, were from 6 normal subjects and from 35 patients with rheumatoid arthritis.

Variations of Metabolism in Synovial Tissues.

There were marked differences in the values obtained for normal and rheumatoid tissues. Table I shows the rate of oxygen uptake (μl. oxygen/mg. tissue nitrogen/hr.), glucose and glocyogen utilisation, and lactate production, by normal and rheumatoid tissue *in vitro*. In the former the rate of oxygen uptake was so low that accurate measurement was impossible under the experimental conditions. In the rheumatoid tissues very marked increases in glucose utilisation and lactate production over the normal values are evident. It was impossible to measure the glycogen content of normal tissue, owing to its low concentration and the small amount of tissue available at one time. In villous tissue, however, the glycogen content could be readily estimated, the mean value being 36 μg. of glucose equivalent/mg. tissue nitrogen. Table I also indicates that the synovial metabolic activity is much less in membrane than in villi, with " fibrotic " villi occupying an intermediate position. In view of this correlation between the metabolic activity and the state of the tissue, the relationship between the above findings and the general or local state of the disease was investigated. As indications of the clinical state at the time of operation, we noted the total duration, the total number of joints involved, the erythrocyte sedimentation rate (Westergren) and the haemoglobin. No correlation between tissue metabolism and any of these factors was apparent.

TABLE I.—*Metabolic Comparison of Normal and Rheumatoid Tissue*

		Rheumatoid tissue.		
	Normal tissue.	Villous.	Fibrotic villous.	Membrane.
$Q_{O_2}(N)$	Trace (10)	27·5 (33)	11·5 (21)	4·7 (26)
		S.E. 1·2	S.E. 0·7	S.E. 0·7
Glucose utilisation μg./hr./mg. nitrogen	46·8 (4)	292·0 (14)	174·0 (14)	95·0 (13)
	S.E. 3·6	S.E. 24·0	S.E. 22·0	S.E. 16·0
Lactate production μg./hr./mg. nitrogen	14·4 (4)	88·0 (8)	47·0 (8)	10·8 (7)
	S.E. 1·3	S.E. 16·6	S.E. 3·8	S.E. 5·1
Glycogen utilisation μg. glucose eqv. /hr./mg. nitrogen	—	9·0 (15)	—	2·7 (8)
		S.E. 0·9		S.E. 0·6

S.E.—The standard error of the mean.
Figures in brackets are the number of experiments.
$Q_{O_2}(N)$.—μl. oxygen utilised per hour per mg. tissue nitrogen in an atmosphere of oxygen.

A local assessment was made similarly with respect to the duration of the disease in the affected joint, and the width of the joint space on anterior/posterior

X-ray, diminution in width being designated minimal, moderate, or marked. Though the local duration of disease showed no significant correlation, the local condition of the joint as shown by X-ray findings, appeared to be related to the metabolic state of the tissue obtained from that joint, the most actively metabolising tissues being found where loss of joint space was minimal. This association was well exemplified in a case in which bilateral synovectomy was performed. The operations were performed within three weeks of each other and throughout this period there was no apparent change in the general clinical state of the patient. The tissue from the left knee, in which there was moderate loss of joint space, was classified as a fibrotic villous, the $Q_{O_2}(N)$ was found to be 11·1. The tissue from the right knee, which had a minimal loss of joint space, was villous and its $Q_{O_2}(N)$ was 35·5.

Effect of Substrates on Metabolic Activity

Table II shows the effect of glucose on the metabolic activity of normal and rheumatoid tissue as expressed by $Q_{O_2}(N)$, lactate production and glycogen utilisation. The presence of glucose did not appreciably affect the rate of oxygen uptake in any synovial tissue. Lactate production by normal and villous tissues was approximately doubled in the presence of glucose, and in villous tissue the rate of glycogen utilisation was halved by the presence of this substrate.

TABLE II.—*Effect of Glucose on Metabolism of Synovial Tissue*

	Normal tissue.		Rheumatoid villi.		Rheumatoid membrane.	
	No glucose.	Glucose.	No glucose.	Glucose.	No glucose.	Glucose.
$Q_{O_2}(N)$	Trace	Trace	30·0	32·0	4·9	4·7
Lactate production μg./hr./mg. nitrogen	7·2	14·4	38·0	88·0	—	10·8
Glycogen utilised μg./hr./mg. nitrogen	—	—	21·6	9·0	—	2·7

In the absence of any substrate, we have found that some actively metabolising villous tissues were capable of metabolising endogenous lactate. To investigate this effect further, we have been able to show that a particular villous tissue, when depleted by pre-incubation for 1 hr. at 37° in the absence of a substrate, metabolised lactate, whereas the same tissue without pre-incubation and under the same conditions did in fact produce small quantities of lactate. The values

EXPLANATION OF PLATE

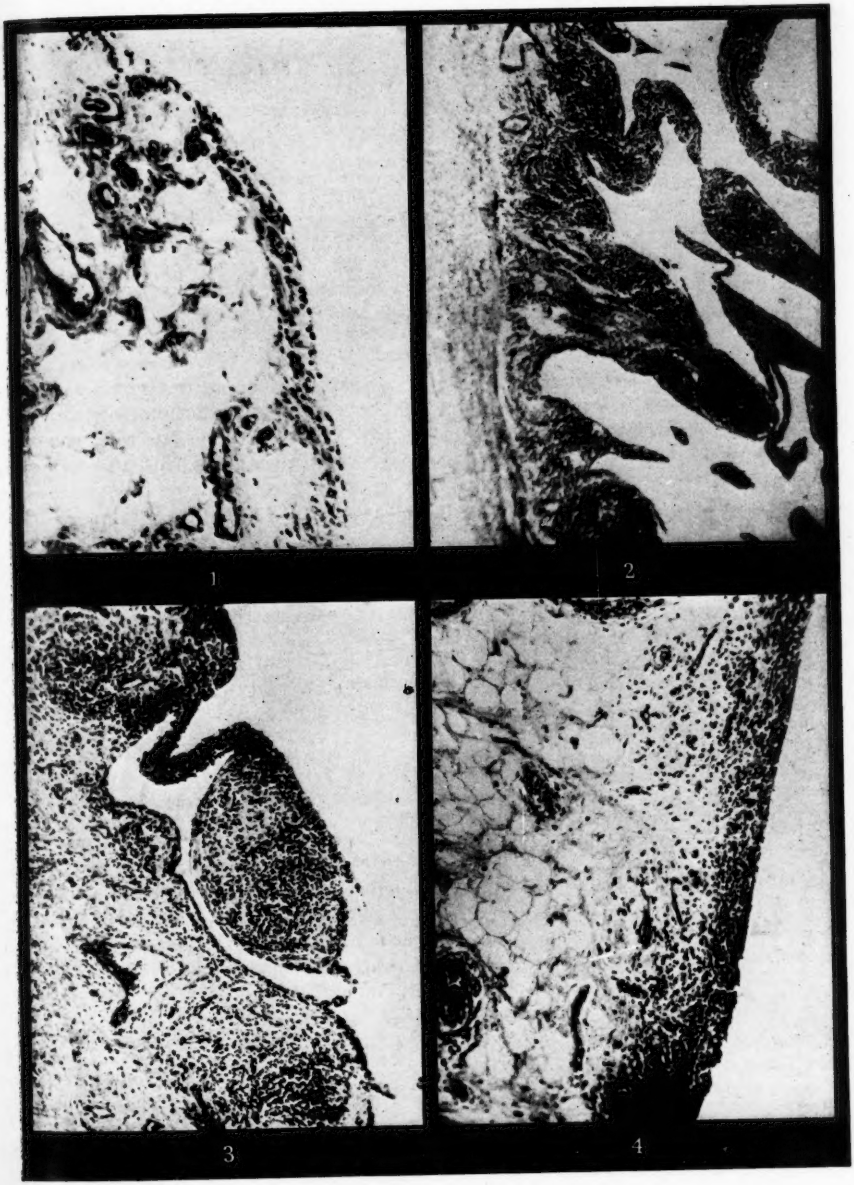
FIG. 1–4 show the typical histological appearances of the four selected classes of synovial tissue. All sections were stained with haematoxylin and eosin.

FIG. 1.—"Normal" synovial tissue. × 200. Traumatic aetiology. Residual capillary engorgement is present.

FIG. 2.—"Villous" rheumatoid synovium. × 50. The section shows a proliferative villous synovitis and a dense subsynovial round cell infiltration.

FIG. 3.—"Fibrotic villous" rhenmatoid synovium. × 150. The villous process is adherent at both ends and the round cell accumulations are less dense.

FIG. 4.—"Membrane" from rheumatoid synovium. × 150. The synovial lining is somewhat necrotic and a round-cell aggregation is present. A few plasma cells are scattered throughout the subsynovium.



Dingle and Page Thomas.

were the production of 20·7 μg./hr./mg. N for the non-depleted tissue, and the utilisation of 58·5 μg./hr./mg. N in the depleted tissue.

The lower values of oxygen uptake in the fibrotic villi and membrane of the rheumatoid group might be ascribed to diminished levels of enzymes or cofactors necessary for oxidative metabolism. That this was not entirely the case, was shown by the addition of various members of the tricarboxylic acid cycle, since all those added considerably increased the rate of oxygen uptake, indicating that the various enzyme systems were capable of stimulation. For example in a rheumatoid membrane with a relatively low Q_{O_2} of 2·1, sodium pyruvate raised this to 3·7 and sodium succinate to 4·3.

Comparison of Synovial Metabolism with that of Other Tissues

Table III shows the Q_{O_2} (N), $Q_{\frac{O_2}{LA}}$, $Q_{\frac{N_2}{LA}}$ and the aerobic and anaerobic glucose utilisation of normal and rheumatoid synovia, and also compares them with horse synovium and other tissues of interest. Normal tissue showed a marked Pasteur effect, which was not nearly so well shown by rheumatoid tissue, the aerobic glycolysis of which was comparatively high. In both tissues there was marked economy of glucose utilisation under aerobic conditions.

TABLE III.—*Aerobic and Anaerobic Metabolism of Synovial and Other Tissues*

Tissue.	Q_{O_2}	$Q_{\frac{O_2}{LA}}$	$Q_{\frac{N_2}{LA}}$	Carbohydrate utilised μg./hr./mg. dry wt. O_2	Carbohydrate utilised μg./hr./mg. dry wt. N_2	Authors.
Normal synovium	Trace	0·4	0·7	4·3	7·2	Present authors
Rheumatoid villi	2·0	1·5	1·9	27·1	44·7	Ditto
,, membrane	0·5	0·3	—	9·5	—	,,
W.II	1·2	1·6	—	11·9	—	,,
Normal horse synovium	0·8	1·7	2·3	—	—	Bywaters (1937)
Rat thymus lymphocytes	10·0	2·0	—	—	—	Miller (1954)
Lymph node, human	4·0	2·0	6·0	—	—	Greenstein and and Meister (1952)
Embryo rat	11·0	3·0	12·0	27·0	48·0	Dickens (1952)
Brain cortex, rat	12·0	1·5	18·0	22·0	72·0	Dickens (1952)
Skin, guinea-pig	1·3	0·8	—	6·4	—	Cruickshank and Trotter (1956)

Q_{O_2} = μl. O_2 uptake/hr./mg. dry weight.
$Q_{\frac{O_2}{LA}}$ = μl. lactic acid equivalent/hr./mg. dry weight, aerobically.
$Q_{\frac{N_2}{LA}}$ = μl. lactic acid equivalent/hr./mg. dry weight, anaerobically.

It is apparent that the values given by Bywaters (1937) for normal horse synovium are much more comparable to our levels for rheumatoid tissue than to normal tissue. The suppression of glycolysis by oxygen in both cases was approximately the same.

In view of the lymphocytic aggregations present in the subsynovium in rheumatoid arthritis, the part that these cells may play in the quantitative metabolic differences between normal and rheumatoid tissue must be considered. In view of this we have included in Table III some values for human lymph nodes and rat thymus lymphocytes. Of interest in this connection was experiment W.II,

(Table III) which was a villous tissue not histologically characteristic of rheumatoid arthritis, as no subsynovial aggregations of round cells were present. The oxygen uptake and lactate production of this tissue were comparable to that of rheumatoid villi, though aerobically the glucose utilisation was somewhat lower.

It may be noted that tissues comparable to rheumatoid tissue in glucose utilisation, both aerobically and anaerobically, show much greater glycolysis and oxidative metabolsim, *e.g.*, embryo rat and rat brain tissue. It would appear that there was a considerable amount of glucose utilised by synovial tissue which could not be accounted for by glycolysis or oxidation.

DISCUSSION

Our results indicate that normal synovial membrane is a tissue with an extremely low oxidative metabolism, though its glucose utilisation, aerobic and anaerobic glycolysis are quite appreciable. As its metabolic requirements are presumably those of maintenance, and possibly of secretion, rather than those of proliferation, glycolysis alone may be adequate.

Table I shows that the presence of rheumatoid disease in the synovial membrane is accompanied by marked metabolic changes. The tissue reaction to the disease involves the appearance of considerable oxidative metabolism, as well as an increase in glycolysis. Since the highest rate of metabolism is found in tissues where the proliferation is most intense, and this process must entail considerable synthetic reactions, it is apparent that an increase in glycolysis alone could not supply the increased energy requirements of the tissue.

As the proliferative process wanes and the general reaction becomes more fibrotic, the metabolic activity of the synovium diminishes, as is shown by the values for fibrotic villi. In the later stages of the disease, as in the membranous tissue often obtained from arthrodesis cases, there is still some residual oxidative metabolism, while aerobic glycolysis has returned to approximately normal values. It would appear therefore that the metabolic activity of rheumatoid synovium is related to the local proliferative activity associated with the disease process. This explains the lack of correlation between the metabolic activity and the various expressions of the overall clinical state. It is typified by the patient who had a bilateral synovectomy. Though her clinical state remained unchanged throughout the operative period, the knee with the fibrotic villi and moderate loss of " joint width " showed less oxidative metabolism than the other.

In considering the increased metabolism of villous tissue, it was important to decide what proportion of the increased metabolism was due to the subsynovial lymphocytic aggregations, rather than to the proliferating synovium. In this connection some of the values given in Table III are pertinent. The glycolytic rate for rheumatoid synovial villi is similar to that found by Miller (1954) for rat thymus lymphocytes, though the latter had a higher oxygen uptake. The results for human lymph nodes are also similar to those for villi. Lymphocytic aggregations could therefore play a large part in the increased metabolism of rheumatoid tissue. An observation which however tends to minimise their part is shown in Table III. In this experiment (W.II) the oxygen uptake and aerobic glycolysis were very similar to those of rheumatoid villous tissue, though the glucose utilisation was somewhat lower. This patient's villi were devoid of any round cell foci.

Table III also illustrates some values of glucose utilisation by rheumatoid tissue. Aerobically and anaerobically the villi show a sixfold increase over the values for normal tissue. The fate of this glucose in synovial tissue merits consideration since a large percentage cannot be accounted for. It is unlikely that this loss of glucose can be due to glycogen synthesis, as we have shown that in the presence of glucose a slight diminution of the glycogen content occurs in villous tissue. In this connection Table II shows that considerably more glycogen is utilised in the absence of substrate. The apparent loss of glucose might, however, be due to the formation of metabolites other than lactic acid.

It is evident that proliferating tissue will utilise glucose, glycogen and sometimes lactic acid, to supply its metabolic needs *in vitro*. Tissue proliferation must involve the provision of considerably more energy than is required for maintenance. Since oxidative metabolism provides more energy-rich bonds than does glycolysis, it is very probable that the marked increase in oxidative metabolism during the proliferative phase of the disease is connected with the provision of the necessary energy. If this assumption is correct it provides at least a partial explanation for the intra-articular action of hydrocortisone. In a previous paper we showed that the presence of hydrocortisone markedly inhibited the oxygen uptake by synovial villi, *e.g.*, using a concentration of 500 μg./ml. (within the therapeutic range) an initial Q_{O_2} (N) of 25·0 was lowered to 7·0. With membrane, under similar conditions, the Q_{O_2} (N) was lowered from 2·5–2·0 (glucose 1 mg./ml. was present in both instances). This inhibition of oxygen uptake must deprive the villi of a considerable part of the energy necessary for tissue proliferation. In the case of the membrane the deprivation of energy is very small by comparison. These observations are compatible with clinical experience of intra-articular hydrocortisone, which appears to be most effective when the proliferative phase is most marked.

SUMMARY

A metabolic comparison between normal and rheumatoid synovial tissue has been made with respect to oxygen uptake, carbohydrate utilisation and lactate production.

The relationship of the metabolic activity of the synovium to the disease state is discussed.

The possible importance of this relationship to the mode of action of hydrocortisone is considered.

We are grateful to Dr. G. D. Kersley for his interest and encouragement and we wish to acknowledge the kind co-operation of the Orthopaedic Staff of the Bath Clinical Area for supplying us with the tissue specimens and Mr. Griffin for the microphotographs.

REFERENCES

Bywaters, E. G. L.—(1937) *J. Path. Bact.*, **44**, 247.
Cruickshank, C. N. D. and Trotter, M. D.—(1956) *Biochem. J.*, **62**, 57.
Dickens, F.—(1952) 'The Enzymes.' Ed. J. B. Sumner and K. Myrbäck, vol. II, p. 675. New York (Acad. Press).
Greenstein, J. P. and Alton Meister—(1952) *Ibid.*, vol. II, p. 1146.
Miller, Z.—(1954) *J. biol. Chem.*, **208**, 327.
Page Thomas, D. P. and Dingle, J. T. M.—(1955) *Brit. J. exp. Path.*, **36**, 195.
Umbreit, W. W., Burris, R. H. and Stauffer, J. F.—(1951) 'Manometric Techniques and Tissue Metabolism,' p. 45. Minneapolis (Burgess Publ. Co.).

VARIATION IN THE TOXICITY OF LECITHINASES (α-TOXIN) FROM DIFFERENT STRAINS OF *CLOSTRIDIUM WELCHII*

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EVANS (1943*a*, *b* ; 1947) showed that the α-toxin is the factor of prime importance in the production of gas gangrene in guinea-pigs infected with *Clostridium welchii*, as the animals were protected by α-antitoxin but not by antibody to other factors such as θ-toxin, hyaluronidase and collagenase. Evans also showed (1945) that the virulence of a strain of *Cl. welchii* was in general related to its capacity to produce α-toxin *in vitro* on the particular medium used ; strains producing small amounts of α-toxin did not cause fatal infections while the majority of those with greater toxigenic capacity readily did so. However, there were some discrepancies in this general correlation ; for instance, two strains, 3893 and 5053, which produced little toxin *in vitro* were highly virulent, while two other strains, 3893 and A102, of similar toxigenic capacity, were avirulent.

Miles and Miles (1950) showed that, in enzymically equipotent doses, lecithinases in the culture filtrates of three strains of *Clostridium bifermentans* were not only much less toxic to mice than *Cl. welchii* lecithinase (α-toxin) but differed from each other in the degree of toxicity, being respectively 9, 60 and 75 times less toxic. The possibility that there may be a similar difference in the toxicity of lecithinases from different strains of *Cl. welchii* has now been examined, by assessing the lethal and haemolytic activity of the toxins in relation to the lecithinase activity towards aqueous lecithin.

MATERIALS AND METHODS

Strains of Cl. welchii

Strains 3983, 3985, 5053 and A102, selected because of their difference in virulence, were obtained from Dr. D. G. Evans. The cultures were kindly examined by Miss M. E. Ross, who confirmed that they were all *Cl. welchii* Type A. Strain S107, which is highly toxigenic, is used for routine production of toxin in this Institute.

Preparation of concentrated toxins

Horse meat infusion was prepared by heating minced muscle (1 lb.) with water (1 litre) to 90° at pH 6·8 and filtering immediately. Peptone (Evans, 3·5 per cent) was added to the extract and after removal of phosphates Na α-glycerophosphate (0·75 per cent) was added and the pH brought to 7·8. A thick layer of the minced extracted muscle was placed in 5 l. flasks and 4 l. of medium added. The flasks were autoclaved at 20 lb. pressure for 30 min., and allowed to cool to 40° ; sterile glucose was added to give a final concentration of 0·6 per cent and inoculation carried out immediately.

The *Cl. welchii* strains were maintained in cooked-meat broth and then subcultured 3 times over a period of 24 hr. in large tubes of infusion-peptone-glycerophosphate broth (50 ml.) without glucose. When the last subculture had grown for 6 hr. at 35° the flasks of

medium were inoculated by adding the whole of one tube to each flask. The flasks were incubated for 9 hr. at 35°, the meat and organisms removed by filtration through paper with the addition of a filtering aid (Stellafilt SSS), and the filtrate precipitated with ammonium sulphate (600 g. per l. of filtrate). The toxin rose to the surface and was removed and dialysed for 3 days against running water at 4°. The solution was reprecipitated with an equal volume of saturated ammonium sulphate, the precipitate centrifuged down and again dialysed against tap water in the cold. It was then centrifuged and the supernatant dialysed against glycerol for 7 days at 4°.

The filtrates from strains 3893, 5053 and A102 all contained large amounts of fatty material which remained in the supernatant when the toxins were precipitated and was rejected without further investigation.

Two toxins from S107 were also examined: WK_4, a concentrated but crude toxin, similar to those above, prepared by dialysis of the precipitate obtained from the culture filtrate with ammonium sulphate; WHR/G, a purified preparation obtained from WK4 by a series of fractional precipitations with methanol at low temperatures.

Assays

Toxicity.—The LD_{50} was estimated by i.v. injection of 0·5 ml. diluted toxin in groups of 5 mice, and the values calculated by the Reed and Muench method. Guinea-pigs were injected with 0·5 ml. diluted toxin into the veins of the forefeet after depilation; two animals were used at each level.

Lecithinase activity.—The toxin (1·0 ml.) diluted in borate buffer (Palitsch), pH 7·4, containing 0·004 M-$CaCl_2$ was incubated for 15 min. at 38° with 1·0 ml. (1·5 mg. P) of an aqueous emulsion of egg lecithin; 1·0 ml. 20 per cent trichloroacetic acid was added and the amount of acid-soluble P in the filtrate determined, with appropriate controls. The activity is expressed in enzyme units (e.u.), one unit being the amount liberating 100 μg. P in these conditions.

Antigen content.—α-Antigen was measured by the combining power with an Elstree *Cl. welchii* (Strain S107) horse antitoxin which had been standardised in terms of international units (i.u.) against standard antiserum obtained from the Department of Biological Standards, National Institute for Medical Research. The combining power was measured in three ways by estimating the L + /10 dose in mice, *i.e.*, the amount still containing 1 LD_{50} after combination with 0·1 i.u. antitoxin; the Le/10 dose enzymically, *i.e.*, the amount still containing 0·5 e.u. of lecithinase activity after combination with 0·1 i.u. antitoxin at room temperature for 30 min.; the Lo/10 dose enzymically, *i.e.*, the largest amount which showed no lecithinase activity after combination with 0·1 i.u. antitoxin. The values are expressed as no. of doses/ml.

θ-Antigen was measured by the combining power with the antitoxin R 5384 designated in the Wellcome Laboratories as containing 150 θ units/ml., using as indicator the haemolysis of sheep or guinea-pig red cells in the absence of Ca ions and after activation with thiolacetate.

κ-Antigen (collagenase) was measured by the combining power with the Wellcome Antitoxin EX1468 designated as containing 110 κ-units/ml., using as indicator an azocoll test similar to that of Bidwell and van Heyningen (1948).

Haemolytic activity.—Guinea-pig red cells, obtained usually from a pool of blood from several guinea-pigs bled into citrate, were washed three times with 0·15 M-NaCl. The cells were stored at 2°, and could be used for 2–3 days, freshly washed. For the test, 1·0 ml. toxin, diluted in borate (Palitsch) buffer pH 7·4, containing additional NaCl 0·17 per cent and 0·004 M-$CaCl_2$, was incubated with 1·0 ml. of the desired concentration of cells in 0·15 M-NaCl at 38°, usually for 30 min. After addition of 5·0 ml. warm saline containing 10 i.u. *Cl. welchii* antitoxin, the mixtures were centrifuged for 4 min. and the haemoglobin in the supernatant was measured in an EEL colorimeter using a green filter. The reading, less that of the blank for the same cell concentration incubated without toxin, is expressed as arbitrary Hb units. Toxins containing θ-toxin were first diluted in Ca-free buffer and preneutralised with an anti-θ rich, anti-α poor serum; two further dilutions were then made, the first in the usual Ca buffer for the assay of haemolysis and the lecithinase activity and the second in Ca-free buffer, to check the absence of θ-toxin activity.

The results therefore are a measure of the haemolytic activity of the lecithinase.

RESULTS

Toxigenic capacity of different strains

It was intended to study the quality of the toxin produced, not the toxigenic capacity of different strains nor the effect of different media. However, the toxicity of the fresh culture filtrates to mice, and in some cases the antigen content, were determined and since the values are of some interest they are set out in Table I, together with some of the data of Evans (1945). The significant points, in relation to the virulence of the strains, are that in our conditions the avirulent strain A102 produced less toxin relatively to strain 3895 than on Evans' medium and the highly virulent strain 5053 produced considerably more.

TABLE I.—*Toxin Production by Various Strains of* Cl. welchii.

		Toxin production *in vitro* (units/ml. culture filtrate).			
			Elstree, 1955†		
Strain.	Virulence in guinea-pigs (Evans, 1945).	Evans, 1945 (L.V. units).*	LD_{50}.	L + /10.	θ-toxin.
S107	Virulent	1·60	150–200	—‡	+
3893	Highly virulent	0·20	40	3·3	+
3895	Avirulent	0·15	14	6·6	*nil*
A102	,,	0·15	10	<2·5	+
5053	Highly virulent	0·05	33	4·0	*nil*

* Peptone-salt-beef extract medium. Values in units of *Cl. welchii* antitoxin (i.u.) required to neutralise the α-toxin in 1·0 ml. supernatant, by the lecitho-vitellin test.

† Meat medium. L + /10 = dose lethal after combination with 0·1 i.u. antitoxin.

‡ S107 usually produces 3–5 L.V. units on the Elstree medium.

Relative toxicity of lecithinases from different strains

Table II shows the toxicity in mice, the lecithinase activity and the antigen content of the concentrated toxins from the five strains. The LD_{50} in guinea-pigs (250 g.) was also determined and was about 5–6 times that for mice (20 g.), *i.e.*, on a weight basis guinea-pigs were above twice as susceptible as mice, but there was no marked difference in relative susceptibility with toxins from different strains of *Cl. welchii*.

TABLE II.—*Properties of Concentrated Toxins from Different Strains*

	Antigen content (units/ml.).				Activity.		
	α-toxin.						
Strain.	L + /10.	Le/10.	Lo/10.	θ toxin.	Toxicity mouse (LD_{50}/ml.).	Lecithinase (e.u./ml.).	Ratio. $\frac{LD_{50}/ml.}{e.u./ml.}$
S107	—	3500	—	44	12,000	4860	2·4
3893	155	101	195	29	860	190	4·5
5053	230	210	405	*nil*	1900	525	3·6
3895	680	640	1200	*nil*	1700	1090	1·56
A102	45	35	76	16	300	88	3·4

The ratio of the toxicity to the *in vitro* enzyme activity varies significantly, from 1·56 in the avirulent strain 3895 to 4·5 in the virulent strain 3893. The ratio for the avirulent strain A102 was, however, nearly as high as those for the highly virulent ones. It is generally accepted that in Type A toxins the killing power is

due to the lecithinase activity and from the concordance of the relative antigen titres by biological and *in vitro* tests, shown in Table II, there is no reason to suppose that this was not the case in these toxins. The difference in the ratios implies that the *in vivo/in vitro* activity of *Cl. welchii* lecithinase can vary in different strains of the organism, although both activities are neutralised by *Cl. welchii* antitoxin. The validity of this conclusion is confirmed by the fact that the differences in biological potency are paralleled by differences in the haemolytic potency of the lecithinases.

Relative haemolytic potency of lecithinases from different strains

It was suggested previously (Macfarlane, 1950) that differences in haemolytic activity might be due to the " goodness of fit " between a particular enzyme and the cell surface of a particular species and also (Macfarlane, 1955) that the degree of " affinity " might be judged from the relative rates of haemolysis at decreasing concentrations.

TABLE III.—*Lecithinase and Haemolytic Activity of Toxins* 3895 *and* 5053 *at Varying Substrate Concentrations*

Haemolysis.			Hydrolysis of lecithin.		
Final conc. cells (v/v)	Haemoglobin liberated (Hb units).		Conc. of lecithin (M × 10^2).	µg. P liberated.	
	Toxin 3895.	Toxin 5053.		Toxin 3895.	Toxin 5053.
8	62	90	2·5	81	81
4	29	58	1·8	81	75
2	10	25	1·25	72	72
1·3	7	17	0·63	57	60
1·0	6	16	—	—	—

Haemolysis test. 1·0 ml. toxin (1·3 e.u.), 1·0 ml. guinea-pig red cells, 20 min. at 38°.
Lecithinase tests. 1·0 ml. toxin (1·3 e.u.), 1·0 ml. lecithin, 8 min. at 38°.

Table III shows the results of an experiment in which the hydrolysis of lecithin and the haemolysis of guinea-pig red cells at varying substrate concentrations by the same number of enzyme units of toxins 3895 and 5053 were measured simultaneously. The lecithinase activities were equal at all substrate levels, but the haemolysis with toxin 5053 was greater than that with 3895.

The differences in haemolytic potency at low cell concentrations were more marked with low concentrations of lecithinase or low concentrations of calcium ions. Many experiments were carried out with simultaneous tests of several toxins in a variety of conditions, in an attempt to find some way in which these differences in the quality of the toxin could be given some quantitative expression characteristic for a strain, as for instance by the calculation of an apparent Michaelis constant. Apart from the fact that the system is heterogeneous, the haemolysis is a secondary phenomenon following the hydrolysis of the lecithin, subject to some lag, and is obviously affected by the fragility of the particular cells used. The main experimental difficulty is that different batches of cells vary in susceptibility to a given concentration of enzyme in an unpredictable manner ; the amount of haemoglobin liberated by the least potent toxin may be so small, or alternatively that liberated by the most potent one be so large, that the

extreme values were inaccurate or not valid even as an approximate measure of initial velocity. The alternative method of estimating the lecithinase activity towards the cell by extracting the phospholipid and estimating the amount decomposed, as was done by Macfarlane (1950), is impracticable with small concentrations of cells.

Although the variability of a red cell test system seems to preclude the establishment of a constant, similar to the Michaelis constant, characteristic for a particular lecithinase, the charting of the haemolytic activity of enzymically equipotent amounts at varying cell concentrations gives a useful indication of the relative affinity. The results of two separate experiments are given in Table IV, and the plot for the mean values of 1/v against 1/s is shown in Fig. 1. It is

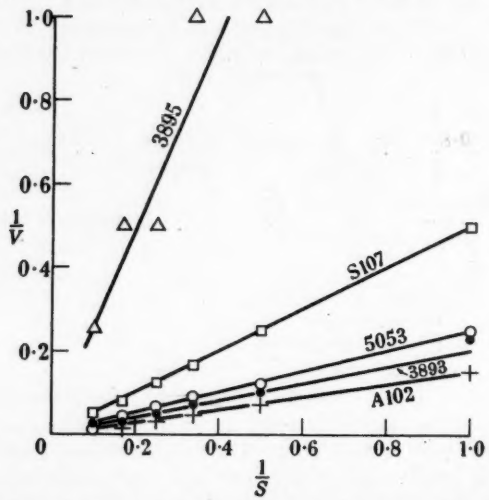

FIG. 1.—Relative haemolytic potency of *Cl. welchii* lecithinases. Data from experiments (*b*) and (*c*), Table IV. $1/s$ = reciprocal of cell conc. (per cent v/v). $1/v$ = reciprocal of Hb units liberated in 30 min. at 38°.

apparent from the slopes that the affinities for guinea-pig red cells of the lecithinases from strain 5053, 3893 and A102 are very similar, and much greater than that of the enzyme from strain 3895 ; the S107 lecithinase is intermediate. The order of haemolytic potency or affinity thus parallels the order of lethal potency in relation to enzymically equipotent amounts of enzyme shown by the ratios in Table II.

Fig. 2 is a similar plot showing the relative potency of the *Cl. welchii* lecithinases 5053 and 3895 and the *Cl. bifermentans* lecithinase BL19. Miles and Miles (1950), using a low concentration of cells and without added calcium ions, found that the *Cl. bifermentans* lecithinase hardly attacked any red cells except those of the mouse. This enzyme, however, is activated by calcium (Lewis and Macfarlane, 1953) and in the presence of calcium and with higher concentrations of cells it lyses guinea-pig and human red cells, though the potency is much lower than that of *Cl. welchii* lecithinases.

TABLE IV.—*Haemolytic Activity of Enzymically Equipotent Amounts of Different Lecithinases*

Cell concentration (per cent v/v).	Toxins (Exp. *a*).				Toxins (Exp. *b*).			
	3895	5053	3893	A102	5053	3893	A102	S107
10	4	60	>90	>90	24	47	>90	19
6	2	34	57	57	18	26	55	13
4	2	22	37	34	12	15	27	8
3	1	14	22	22	9	11	19	6
2	1	10	13	14	7	8	12	4
1	1	4	6	7	4	3	6	2

Enzyme concentration 0·2 e.u./ml.; incubation 30 min. at 38°. Haemolysis in Hb units.

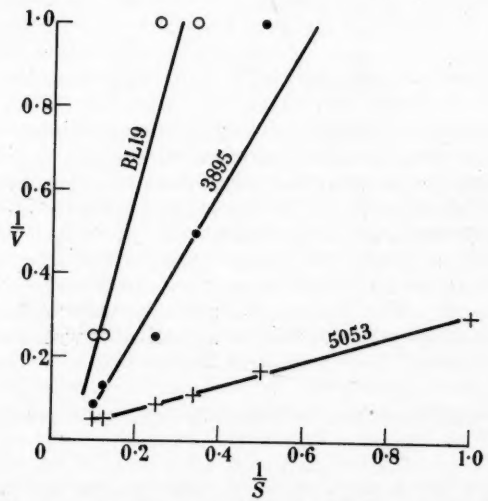

FIG. 2.—Relative haemolytic potency of *Cl. welchii* and *Cl. bifermentans* lecithinases. Enzyme concentration 0·1 e.u./ml. 60 min. at 38°. s = cell concentration per cent v/v. v = haemolysis in Hb units.

Search for possible factors influencing the haemolytic effect of the lecithinase

No evidence was found that the toxins tested contained in addition to the lecithinase any factor which could directly cause haemolysis other than the θ-toxin. Test doses of the toxins, pre-neutralised with θ-antitoxin, were combined with graded amounts of α-antitoxin, and the residual lecithinase and haemolytic activities determined; the two activities were always inhibited in parallel. It is easy to postulate, and impossible to exclude absolutely, the existence in some strains of an accessory factor which by some prior alteration of the surface of the cell potentiates (or inhibits) the approach of the lecithinase to its substrate. It is probable that such a factor would already have been recognised in one or other of the laboratories which have worked on the properties or the purification of the α-toxin; in fact the haemolytic, enzymic and toxic effects of the α-toxin have been

found inseparable. The following experiments were however made in search of some accessory factor.

Effect of purification.—The haemolytic activity of a unit of lecithinase in a crude and purified preparation from S107 over a wide range of concentration of cells was found to be the same (Table V) although the ratio of the lecithinase activity to other antigens had increased eightfold.

TABLE V.—*Haemolytic Potency of Crude and Purified Lecithinases from S107*

	Properties of lecithinase preparation.											
	e.u./mg. N.	Ratio of antigen units.			Haemolysis by 0·4 e.u. in 30 min. at cell conc. (per cent v/v).							
		α	θ	κ	10.	6.	4.	3.	2.	1.		
					Hb units liberated							
WK4	1,500	100	90	30	18	10	7	6	4	2		
WHR/G	10,000	100	12	3·5	20	10	6	5	4	3		

Effect of preheating.—A sample of toxin 5053 was diluted in borate buffer, pH 8·0, $CaCl_2$ 0·004 M to a concentration of 24 e.u./ml. and some of this was heated at 58° for 15 min., a treatment which destroys, for instance, the receptor-destroying enzyme (Macrae, 1947). The heated and unheated samples were further diluted to various strengths in borate buffer, pH 7·4, $CaCl_2$ 0·004 M, and the lecithinase and haemolytic potency of these dilutions were determined simultaneously. (This was necessary in case there was loss of lecithinase activity on heating, though none occurred.) The haemolytic potency was unchanged by heating, *i.e.*, no evidence of selective destruction of an activator was obtained.

TABLE VI.—*Additive Effect of Toxins of Different Haemolytic Potency*

	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	
Toxin 3895 (ml.)	1·0	0·5	0	0	0·5	
Toxin 5083 (ml.)	0	0	1·0	0·5	0·5	
Cell concentration (per cent v/v)	Hb units.					Calc. sum of (*b*)+(*d*)
10	42	10	82	68	77	78
6	27	7	72	56	57	63
3	25	9	46	29	34	38
2	17	7	33	24	29	31

Toxins 3895 and 5053 diluted to 0·8 e.u./ml. Samples + buffer to 1·0 ml. + 1·0 ml. cell suspension; 30 min. at 38°.

Synergic effect of toxins of different potency.—Samples of toxin 3895 (low potency) and 5053 (high potency) were diluted separately to a concentration of 0·8 e.u./ml. The haemolytic activity of 1·0 and 0·5 ml. samples separately and of a mixture of 0·5 ml. of each toxin was determined with four concentrations of cells. The results in Table VI show that the activity of the mixture was almost identical with the calculated sum of the components. No evidence therefore for the presence either of an activator in toxin 5053 or an inhibitor in the less potent toxin 3895 was obtained.

DISCUSSION

The experiments reported show that the lethal and haemolytic potency of the lecithinase (α-toxin) of *Cl. welchii* in relation to the enzymic activity towards aqueous lecithin is not constant in all strains of the organism. The ratio of the toxicity in mice to the enzymic activity in a standard *in vitro* test varied nearly threefold in the toxins examined and, by measurement of the haemolysis over a ten-fold range of cell concentrations, equally striking differences were observed in the haemolytic potency. The activities—lethal, haemolytic and enzymic—were all neutralised by *Cl. welchii* antitoxin and no evidence was obtained of the existence of any activator or inhibitor in the different toxins which could account for the effect. It is concluded, therefore, that the differences in potency arise from differences in the configuration of the α-toxin from different strains of the organism, differences apparently too small to affect the degree of neutralisation of the toxin with antitoxin used but sufficient to affect the access of the enzyme to its substrate embedded in the cellular structure.

These results extend therefore to another and a pathogenic species, the finding of Miles and Miles (1950) that the lecithinases of various strains of *Cl. bifermentans* varied in biological potency, and it seems justifiable to suppose that this is a general phenomenon, *i.e.*, that under an immunological umbrella specific for species or type, the molecules of a factor elaborated by organisms have a strain-specific structure which affects their biological potency and hence presumably the virulence of the organism. Conversely, a cell of specific structure can distinguish, by its susceptibility to haemolysis, between two closely related enzymes. The interest of such differentiations in the problems of biological differentiation was discussed previously (Macfarlane, 1950).

The present results resolve to some extent the discrepancies found by Evans (1945) in his survey of the relationship between the virulence of *Cl. welchii* strains and their capacity to produce α-toxin *in vitro*. The α-toxin is an invasive factor and the spread of the disease will therefore be influenced both by the amount of lecithinase produced by the initial inoculum in unit time and by the potency of this lecithinase in producing local necrosis favouring further growth. Thus the avirulence of strain 3895 can be attributed in part at least to the fact that its α-toxin is of low potency. On the other hand, though strain A102 produced a toxin as potent as those of virulent strains, its toxigenic capacity both on Evans' medium and the Elstree medium was low ; in contrast, the strain 5053 displayed on the Elstree medium a toxigenic capacity which, together with the potency of the toxin produced, is compatible with the virulence in guinea-pigs found by Evans.

Differences in the α-toxins were demonstrable because their activity as lecithinases is known and can be measured independently of the host or of any cellular structure which could bias the results. The biological and biochemical activity of other lethal toxins cannot be compared in a similar way until their biochemical action is known.

SUMMARY

Toxins were prepared from five strains of *Clostridium welchii* known to differ in their virulence for guinea-pigs.

The activity of the α-toxin (lecithinase) present was determined by two methods, toxicity to mice expressed as the LD_{50} titre and hydrolysis of aqueous lecithin, expressed in arbitrary enzyme units (e.u.).

The ratio of the biological activity to the enzymic activity (LD_{50}/e.u.) varied from 1·56 for the toxin of the avirulent strain 3895 to values between 3·4 and 4·5 for three toxins, two of which, 3893 and 5053, were derived from highly virulent strains; the third potent toxin was derived from the avirulent strain A102, which appears, however, to have a low toxigenic capacity.

The relative haemolytic activity towards guinea-pig red cells of enzymically equal amounts of the lecithinases was assessed by comparison of the velocities of haemolysis over a range of cell concentrations, and was found to parallel the relative biological potency.

In all the toxins the enzymic and haemolytic activities were reduced in parallel by combination with *Cl. welchii* antitoxin, and no potentiating or inhibiting factor was found which could account for the variation in the biological potency.

It is concluded that the differences in potency are due to small differences in the configuration of α-toxin from different strains.

We are indebted to Dr. D. G. Evans for the strains of *Cl. welchii* and to Dr. G. H. Warrack of the Wellcome Laboratories for the gift of antitoxins.

REFERENCES

Bidwell, E. and van Heyningen, W. E.—(1948) *Biochem. J.*, **42**, 140.
Evans, D. G.—(1943*a*) *Brit. J. exp. Path.*, **24**, 81.—(1943*b*) *J. Path. Bact.*, **55**, 427.—(1945) *Ibid.*, **57**, 75.—(1947) *Brit. J. exp. Path.*, **28**, 24.
Lewis, G. M. and Macfarlane, Marjorie G.—(1953) *Biochem. J.*, **54**, 138.
Macfarlane, Marjorie G.—(1950) *Ibid.*, **47**, 270.—(1955) *Symp. Soc. gen. Microbiol.*, **5**, 57.
Macrea, J. F.—(1947) *Aust. J. exp. Biol. med. Sci.*, **25**, 127.
Miles, Ellen M. and Miles, A. A.—(1950) *J. gen. Microbiol.*, **4**, 22.

THE SPREAD OF A NEUROTROPIC STRAIN OF HERPES VIRUS IN THE CEREBROSPINAL AXIS OF RABBITS

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THE question whether a neurotropic strain of herpes virus spreads within the nervous system along axis cylinders or within tissue fluid spaces was first raised almost simultaneously by Marinesco and Draganesco (1923) and by Goodpasture and Teague (1923–24). The former regarded the intercellular spaces as the more probable pathway for the centripetal movement of the virus, whereas the latter believed that it gained early access to the peripheral nerve fibres and was conducted within the confines of the axons to their motor or sensory nuclei in the central nervous system. Recently Field (1952) has reconsidered the problem by again employing histological methods to identify the sites of inflammation in the trigeminal nerve trunk and the brain stem resulting from the inoculation of the virus into the cornea and masseter muscle of rabbits. From his findings, he has gained the impression that they tend to support the hypothesis of "lymphatic space progression rather than that of axonal transmission."

Few attempts have yet been made to identify sites of experimentally induced inflammation in the central nervous system by making use of the local escape of some recognisably marked colloid in spite of the fact that the normally low permeability of its capillary walls to large molecules renders this organ particularly well suited to the employment of such techniques. In their study on the invasion of the brain stem by herpes virus inoculated into the territory of the fifth cranial nerve, McClellan and Goodpasture (1923–24) injected trypan blue into the circulating blood and observed its extravasation at the site of inflammation. Later, Faber (1936–37) used the same procedure for identifying the sites of pre-paralytic lesions in monkeys in which poliomyelitis had been induced by intranasal infection. Although vital dye techniques display the foci of inflammation conspicuously to the naked eye, any attempt to apply them quantitatively, such as that devised by Sachs and Lummis (1955), has the serious disadvantage that it necessarily entails the destruction of the specimen and thus precludes any subsequent comparative histological examination of the tissues.

The introduction of radioactive isotopes for the labelling of proteins has not only extended the applicability of this method of detecting sites of injury through the focal accumulation of a marked colloid, but it has placed it on a better quantitative basis than any possible with vital dyes. The present paper describes the use of radio-iodinated homologous serum proteins for identifying the location and assessing the severity of the inflammatory reactions excited in the cerebrospinal axis of rabbits by the injection of a neurotropic strain of herpes virus into three widely spaced peripheral spinal nerve trunks. Its conclusions may be anticipated

by stating that the segmental distribution of the subsequent inflammatory reactions in the spinal cord appear to be more compatible with a dispersal of the virus from its point of entry into the neuraxis by way of its tissue fluid spaces rather than along its axis cylinders.

METHODS

Animals

All observations were made on Copenhagen White rabbits weighing 1·5–2·5 kg.

Virus

The Brussels strain of neurotropic herpes virus used was kindly given by Dr. Pierre Lépine. It was maintained by passage in the central nervous system of rabbits, in some by intracerebral inoculation and in others by direct inoculation into the lumbar spinal cord. These latter preparations were the more potent. The infected nervous tissue from the moribund animals was triturated with four times its volume of ice-cold sterile broth and preserved in the freeze-dried state in small ampoules in volumes of 0·1 ml. Re-suspension was effected by the addition of an equal volume of sterile water. When injected intracerebrally, these preparations killed rabbits in 3–6 days.

Operative procedures

Before operation, the rabbits were anaesthetised with "veterinary Nembutal" (0·4 ml./kg.) given intravenously and followed by open ether. The sciatic, median or first lumbar spinal nerves were exposed with aseptic precautions, and the virus suspension was injected directly into the selected nerve trunk with the aid of a micrometer-controlled 0·5 ml. tuberculin syringe. The volumes injected were about 40, 30 and 20 μl. respectively. The wounds were closed with silk sutures and covered with a protective layer of collodion.

Post-operative clinical observations

Each animal was examined at least once daily for evidence of pyrexia, paralysis and changes in knee and "splay" reflexes (see Gutmann, 1942).

Preparation of radioactive protein

Rabbit plasma proteins labelled with ^{131}I were prepared by the method described by Veall, Pearson and Hanley (1955). The protein solution was injected under light Nembutal anaesthesia into the marginal ear vein 20 hr. before the animal was killed. The dosage usually employed was 400 μc./kg. in a volume of 1–3 ml./kg. About 40 per cent of this labelled protein remained in the circulating blood at the end of 20 hr.

Preparation of specimens from the central nervous system

Immediately before death a sample of blood was taken from the marginal ear vein for the subsequent determination of the terminal plasma radioactivity. The rabbits were killed with Nembutal and at once eviscerated. The brain and spinal cord were then exposed *in situ*, and the carcass immersed for 48 hr. in 10 per cent formol-saline. After fixation, the entire central nervous system was removed *en bloc*, care being taken to trim away all extramedullary nervous tissue. The spinal cord between the levels of the lower border of the cerebellum and the third sacral nerve roots was divided into 17 portions of equal length. In most rabbits this mode of division gave portions of cord 2 cm. in length and weighing between 150 and 400 mg. The relationship of these 17 portions to the anatomical segments of the spinal cord was as follows:

Portion number.	Segment.	Portion number.	Segment.	Portion number.	Segment.
1	C 1 and 2	7	T 7 and 8	13	L 3
2	C 3 and 4	8	T 9 and 10	14	L 4 and 5
3	C 5 and 6	9	T 11	15	L 6
4	C 7 and 8; T 1	10	T 12	16	L 7; S 1
5	T 2, 3 and 4	11	L 1	17	S 2 and 3
6	T 5 and 6	12	L 2		

The small terminal portion of the spinal cord (No. 17), the conus medullaris, yielded radioactivity values both higher and more variable than those of the remaining sixteen—a difference attributable to the proportionately larger contribution of irremovable nerve rootlets at this level. For this reason, this portion was discarded.

After the separation of the cerebral hemisphere and cerebellum, the rest of the brain was divided into four roughly equal portions: the remainder of the medulla oblongata (M), the pons (P) and the regions of the corpora quadrigemina (Q) and the basal ganglia (B).

Each specimen, after light blotting with filter paper, was weighed promptly with particular care to avoid desiccation and placed immediately in a test tube (80 × 12 mm.) containing 4 ml. of formol-saline.

Estimation of radioactivity

Measurements of gamma-ray activity were made with an apparatus of the type described by Veall and Baptista (1954), modified for use with a smaller specimen by reducing the number of G4Pb tubes to four and mounting them symmetrically as closely as possible to the centrally placed sample tube. This apparatus had a sensitivity for ^{131}I of 7500 c.p.m./μc., and a background of 20 c.p.m.

Each specimen was counted for two periods of 5 min. each, and an average value obtained. From this and from the weight, the corrected c.p.m./g. was calculated. In order to achieve comparability between the various animals used, this latter figure was in each case related to the corresponding terminal level of plasma radioactivity. Thus, by dividing the c.p.m./g. for each specimen by the plasma c.p.m./100 μl. obtained for the respective animal, an index, the "plasma equivalent," was derived. In this way, allowance was made for the individual differences in the plasma radioactivity of the various rabbits.

RESULTS

Control Animals

The levels of radioactivity developing in the various designated portions of the central nervous system as a result of the intravenous injection of the labelled plasma proteins were determined for four rabbits. From Fig. 1, it can be seen

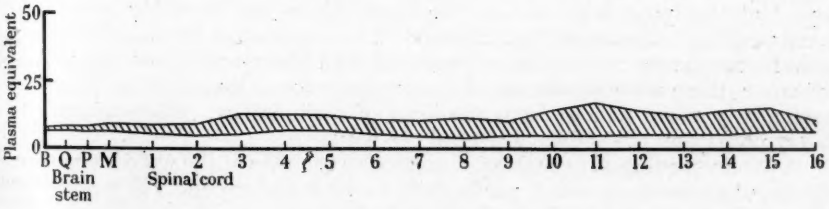

FIG. 1.—Limits of radioactivity (expressed as plasma equivalents) of consecutive portions of the brain stem (B., Q., P. and M.) and spinal cord (No. 1 to 16) of four control rabbits which had been injected intravenously 20 hr. before death with ^{131}I-labelled homologous plasma proteins.

that there was little variation in the plasma equivalent amongst the twenty consecutive portions into which the neuraxis from the basal ganglia to the lumbar enlargement had been divided, and that their radioactivity could be accounted for by a contained amount of plasma equivalent to about 1 per cent of their volume. Since the capillaries of the central nervous system are normally relatively impermeable to colloids, it seems likely that the greater part of these labelled proteins was present in the small blood vessels whose contents were less likely than those of

the larger and more superficial ones to be drained during the process of preparing the specimens.

Infected Animals

Clinical course

Infected rabbits usually developed pyrexia of from 104·5–106·5° F. (rectal temperature) on the 5th or 6th day after an intraneural inoculation. Nervous disturbances appeared 1–3 days later. After an inoculation into the median or sciatic nerves, paresis of the injected limb was invariably the first sign of involvement of the spinal cord. Soon, the opposite limb was also affected, and the full syndrome of fore- or hind-quarters paralysis quickly followed. After an injection into the first lumbar spinal nerve, paresis of the limbs appeared late and always initially in the hind limbs; sagging of the belly wall from weakness of the abdominal muscles was sometimes observed. The " splay reflex " described by Gutmann (1942) was usually lost after either a sciatic or first lumbar spinal nerve inoculation, but the knee jerks were consistently abolished only after the injection of the former. Moribund animals were usually markedly hypothermic and showed severe generalised muscular weakness. None of the rabbits injected intraneurally presented the typical syndrome of herpes encephalitis (fits, grinding of teeth and profuse salivation).

The animals were generally killed one, sometimes two or three, days after the onset of definite nervous disturbances: thus the labelled protein was usually given on the day on which unequivocal signs of injury to the spinal cord had become apparent.

Gross appearances

The spinal cords from infected rabbits usually showed superficial haemorrhages distributed in a characteristic manner. The largest were always on the dorsal aspect at the spinal level and on the same side as the incoming sensory roots corresponding to the nerve trunk injected. There were often further smaller superficial haemorrhages in the adjacent portions both above and below that primarily affected; these were sometimes on the opposite dorsal aspect of the spinal cord, particularly those remote from the level of main injury. Haemorrhages were rarely present on the ventral aspect of the cord.

A patchy meningeal exudation, unrelated to the sites of the main haemorrhagic lesions, was sometimes found, particularly in the sacral region. The more severely affected portions were often swollen and friable.

Segmental distribution of radioactivity

The radioactivity determinations of the affected portions of the spinal cord were substantially and consistently raised: at their maximum, the plasma equivalents were some 10–12 times higher than those found for the corresponding portions in the control animals. Moreover, the highest values in each animal were found in the portions segmentally related to the nerve trunk injected. Although the plasma equivalents in the most severely affected portions reached maximal values closely similar for all three sites of intraneural injections used, the curves for the distribution of the radioactivity along the cerebrospinal axis showed characteristics distinctive for each. The individual records for the four rabbits in

each of the three groups injected by the median, first lumbar spinal and sciatic nerves are set out in Fig. 2, 3 and 4 respectively. The mean values for each of the three groups are shown in Fig. 5.

Median nerve.—The central connexions of the brachial plexus lie in portions 3 and 4 of the spinal cord. Of the rabbits injected through the median nerve, the maximal radioactivity was found in portion 3 in two rabbits and in portion 4 in the other two. The mean values for all four rabbits, shown in Fig. 5, indicate that these portions are almost equally affected. From these maxima, the radio-

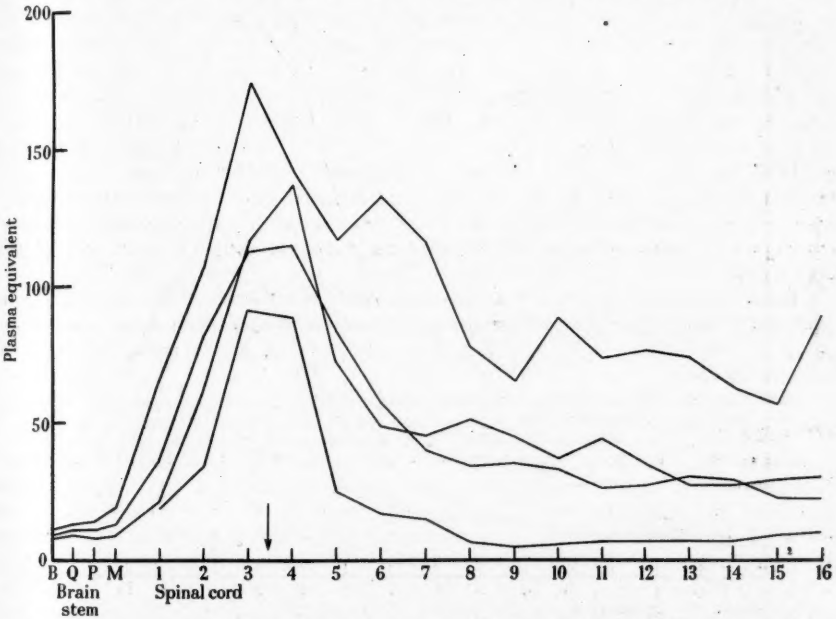

FIG. 2.—Radioactivity of the cerebrospinal axis of four rabbits which had been injected firstly with herpes virus into the median nerve and secondly with ^{131}I-labelled proteins intravenously. The arrow marks the spinal level of the nerve inoculated.

activity fell sharply in the cephalad direction, reaching values at the junction of the spinal cord and medulla oblongata that were little larger than those observed in the control rabbits, while in the caudal direction, significantly raised values were found far down in the thoracic and even in the lumbar segments. It would seem therefore that the agent which provokes the inflammation spreads more easily downwards than upwards in this region of the spinal cord. This difference might be associated with the relative fixity of the cord to surrounding structures near the base of the skull and its greater mobility within the vertebral canal in the thoracic and lumbar regions.

First lumbar spinal nerve.—This nerve arises from a segment included in portion 11, and from the curves depicting radioactivity given in Fig. 3, it can be seen that in all four animals in this group the peak lay either in this portion or

in those immediately above or below it. From this maximum, as can be seen from the curve for mean values given in Fig. 5, the fall is almost symmetrical, the abnormally elevated radioactivity extending downwards into the sacral region and upwards into the cervical region as far as the medulla. In this mobile region of the spinal cord, the agent responsible for the myelitis appears to be carried with equal facility in either direction.

Sciatic nerve.—This nerve trunk is composed of elements derived mainly from the 7th lumbar and 1st sacral segments, both of which lie in portion 16 of the spinal

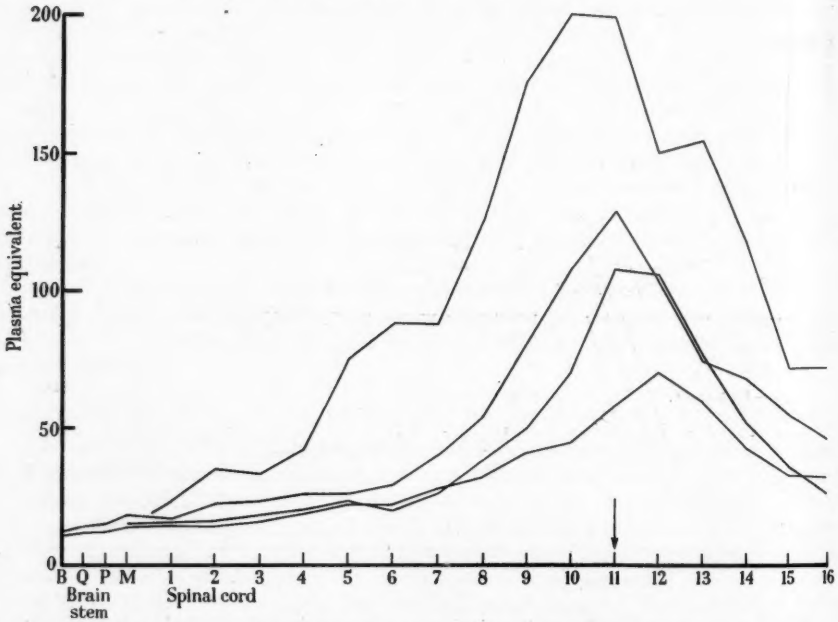

FIG. 3.—Radioactivity of the cerebrospinal axis of four rabbits which had been injected firstly with herpes virus into the first lumbar spinal nerve and secondly with ^{131}I-labelled proteins intravenously. The arrow marks the spinal level of the nerve inoculated.

cord. As can be seen from Fig. 4, the highest radioactivity was usually in this portion, although in one rabbit the maximum occurred two portions higher. It is evident from the mean curve in Fig. 5, that the radioactivity declined in a roughly logarithmic manner in a cephalad direction to reach values in the lower part of the thoracic spinal cord little above those found in the corresponding portions of the control animals.

Histological appearances

In order to compare alterations in radioactivity at various levels with microscopical evidence of structural injury, histological sections were prepared from each portion of the infected spinal cords. In brief it may be stated that the changes

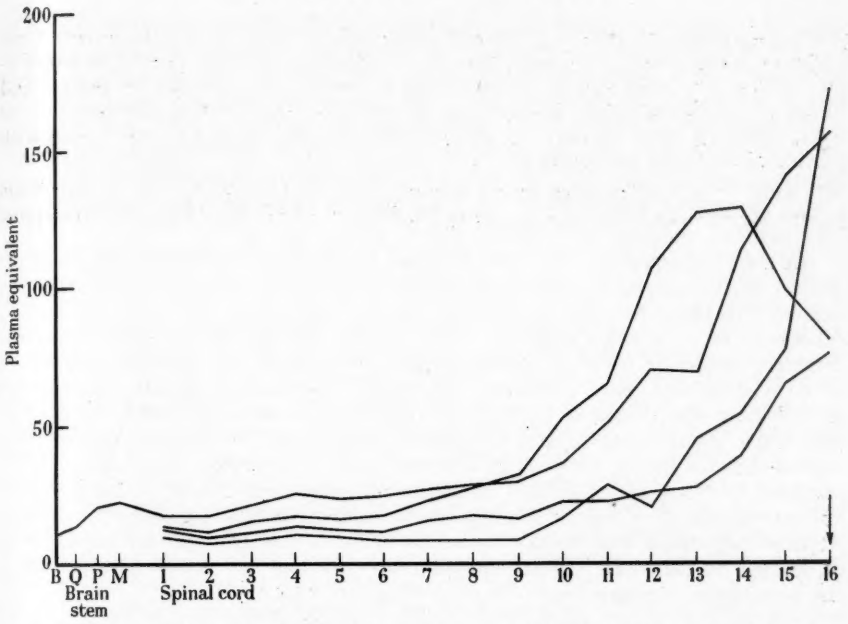

FIG. 4.—Radioactivity of the cerebrospinal axis of four rabbits which had been injected firstly with herpes virus into the sciatic nerve and secondly with ^{131}I-labelled proteins intravenously. The arrow marks the spinal level of the nerve inoculated.

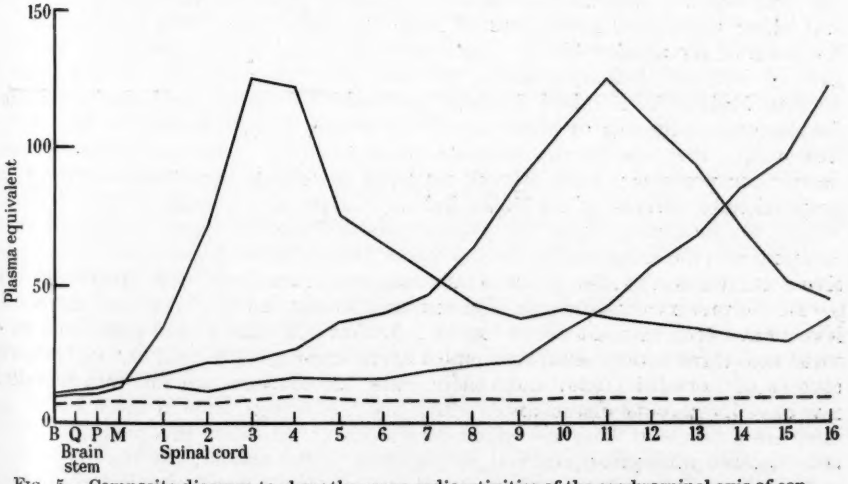

FIG. 5.—Composite diagram to show the mean radioactivities of the cerebrospinal axis of control rabbits (interrupted line) and of three groups of rabbits in which myelitis had been produced by inoculations of virus into the median, first lumbar spinal and sciatic nerves (continuous lines).

observed closely resembled those described by Levaditi (1926) for rabbits with herpetic encephalitis and myelitis. A noteworthy feature of herpes myelitis, however, is the variability in the histology of the lesions. Though the morphology of the predominant lesion was usually the same throughout any particular spinal cord, differing only in severity at different levels, there was often surprising variation between cords taken from animals which had presented essentially the same clinical disturbances. Flexner and Amoss (1925) referred to this histological variability, and noted a " wide disparity of lesions and no close relationship between their intensity and the severity of the symptoms ".

In the present study there was a broad correlation between the height of the radioactivity, expressed in plasma equivalents, and the severity of the morphological changes. In those portions in which the plasma equivalent had been substantially raised—to seven or more times the control value—the lesions were of the advanced form characterised by focal haemorrhages, destruction of neurones, infiltration of cord substance and meninges with many polymorphonuclear, macrophage and plasma cells, vascular cuffing and occasional areas of necrosis. In portions with lower plasma equivalents—roughly twice those of the controls—the morphological signs of injury were slight or even absent. Typically, these milder lesions assumed the form of congestion of the blood vessels, activation of the microglia, and accumulations of macrophages in perivascular spaces and beneath the meninges. At this stage there was usually no involvement of the grey matter nor evidence of damage to neurones. When the plasma equivalent lay between these values, the histological changes were of intermediate severity.

DISCUSSION

It has long been recognised that early in the acute inflammatory reaction, the local capillary endothelium becomes abnormally permeable to plasma proteins and other circulating substances of large molecular size. This modification in the walls of the smaller blood vessels has been found notably useful for locating sites of incipient inflammation in the skin and other organs. In the central nervous system, such raised vascular permeability proves particularly valuable for demonstrating foci of tissue injury by reason of two distinctive features of this organ : first, the almost complete impenetrability of the normal blood-brain barrier for proteins ; and, second, its loose extensible structure and the high proportionate volume of its tissue fluids (Wallace and Brodie, 1939 ; Davson, 1955). Consequently, once the plasma proteins are no longer restrained from escaping into the extravascular tissue spaces, there is little obstacle to their progressive accumulation at sites of acute inflammation in the brain and spinal cord.

In the present experiments, segmental infections of the spinal cord at various levels have been brought about by the injection of a neurotropic strain of herpes virus into three widely separated major nerve trunks. As is well known from the studies of Levaditi (1926) and others, such inoculations are followed within a few days by nervous disturbances which are first recognisable in those structures that are innervated from the spinal cord segment primarily involved, but which soon become widespread and end in the death of the animal. By the injection of radio-iodinated homologous plasma proteins into the general circulation followed by their localised escape thence into the extravascular tissue fluid spaces of the spinal cord, it becomes possible to identify the site of the major injury and to

determine quantitatively the gravity of the damage at all levels of the cerebrospinal axis. In all the animals examined in the present experiments, it was found that the segmental level corresponding to the particular nerve trunk inoculated coincided closely with the level for the peak of the radioactivity, and that from this maximum the latter fell roughly exponentially. This gradation was particularly noteworthy after inoculation of the first lumbar nerve, for with this trunk, the radioactivity of the various portions of the spinal cord was found to be distributed almost symmetrically about the highest value in the first lumbar segment.

It is of interest in connexion with the possible modes of spread of neurotropic viruses in the central nervous system that the distribution of the radioactivity in spinal cords infected at a particular level appears to bear little relationship to the intraspinal architecture of the incoming axons of the posterior spinal nerve roots. Yee and Corbin (1939) traced the centripetal pathways followed by these sensory axons for the first five cervical nerves in rabbits by determining the distribution of the degenerating fibres in the substance of the cord resulting from the surgical division of the individual posterior roots proximal to the spinal ganglion. In no instance did the signs of degeneration extend caudally for more than four segments, which in this region of the cord occupy a length of about 3 cm. Since the median nerve arises mainly from the sixth cervical segment, it is unlikely that its sensory descending collateral axons pass beyond the segment of the spinal cord included in portion five in our experiments. The marked elevation of the radioactivity of portions much caudal to number five, however, shows that the zone of inflammation has extended to segments much below the lowest extremity of the descending sensory collateral axons. It seems noteworthy, moreover, that whereas the intraspinal divisions of the sensory neurones of the median nerve pass mainly upwards, the signs of inflammation in the cord are more extensive in the segments caudal to the level of its primary involvement. The results of the injection of virus into the first lumbar spinal nerve also display a similar lack of agreement between the intraspinal architecture of the incoming sensory axons and the distribution of the elevated radioactivity in the infected cord. It would seem, therefore, that this virus, possibly because of its pantropic properties, becomes dispersed from its level of entry into the spinal cord through its highly orientated tissue spaces rather than intracellularly along axonal pathways.

SUMMARY

Herpes myelitis has been produced at various levels in the spinal cord of rabbits by the injection of a neurotropic strain of the virus into the median, first lumbar spinal or sciatic nerves.

Through the subsequent intravenous injection of ^{131}I-labelled homologous plasma proteins and their preferential accumulation at sites of inflammation, the extent and severity of the resulting myelitis at various segmental levels has been determined.

From the comparative segmental distribution of the radioactivity in the infected spinal cord, it appears that the spread of the virus from its level of entry is better explicable on the basis of dispersion through tissue fluids than along axonal pathways.

The method here employed has provided a better quantitative estimate of the severity of the myelitis caused by this virus than that obtained from histological

studies. It might also prove useful in experiments designed to disclose the initial sites of entry and subsequent dispersion of certain other neurotropic viruses in the central nervous system.

We wish to thank Mr. N. Veall for much help in the course of this work.

REFERENCES

DAVSON, H.—(1955) *J. Physiol.*, **129**, 11.
FABER, H. K.—(1936–37) *Proc. Soc. exp. Biol., N.Y.*, **35**, 10.
FIELD, E. J.—(1952) *J. Path. Bact.*, **64**, 1.
FLEXNER, S. AND AMOSS, H. L.—(1925) *J. exp. Med.*, **41**, 215.
GOODPASTURE, E. W. AND TEAGUE, O.—(1923–24) *J. med. Res.*, **44**, 139.
GUTMANN, E.—(1942) *J. Neurol. Psychiat.*, **5**, 81.
LEVADITI, C.—(1926) 'L'Herpes et le Zona.' Paris (Masson).
MCCLELLAN, R. H. AND GOODPASTURE, E. W.—(1923–24) *J. med. Res.*, **44**, 201.
MARINESCO, G. AND DRAGANESCO, S.—(1923) *Ann. Inst. Pasteur*, **37**, 753.
SACHS, R. A. AND LUMMIS, W. L.—(1955) *Proc. Soc. exp. Biol., N.Y.*, **90**, 565.
VEALL, N. AND BAPTISTA, A. N.—(1954) *Brit. J. Radiol.*, **27**, 198.
Idem, PEARSON, J. D. AND HANLEY, T.—(1955) *Ibid.*, **28**, 633.
WALLACE, G. B. AND BRODIE, B. B.—(1939) *J. Pharmacol.*, **65**, 220.
YEE, J. AND CORBIN, K. B.—(1939) *J. Comp. Neurol.*, **70**, 297.

THE RÔLE OF THE LYMPHATIC SYSTEM IN THE PATHOGENESIS OF ANTHRAX

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WHEN animals die of anthrax there are prominent pathological changes in the lymphatic system. If the primary site of infection is the skin, the regional lymph nodes are enlarged, haemorrhagic and contain living anthrax bacilli. At the primary lesion there is oedema and cellular infiltration, as well as multiplication of bacilli (Cromartie, Bloom and Watson, 1947). If, on the other hand, anthrax is initiated by the inhalation of spores there is less involvement at the site of invasion. Changes in the lung parenchyma have been described, such as hyperaemia, oedema and cellular infiltration in animals sacrificed at various intervals between 12 and 48 hr. after exposure to a dose expected to be fatal (Young, Zelle and Lincoln, 1946), but these are certainly slight and may be absent (Ross, personal communication). Barnes (1947) has shown that bacilli do not multiply in the lung itself, but cause a massive infection of the mediastinal lymph nodes which precedes bacillaemia and death, an observation confirmed by plate counts on a suspension of the nodes. He concludes that the alveolar lining acts merely as a portal of entry of the bacilli, and that multiplication and subsequent invasion of the blood stream takes place only after infection of the lymph nodes draining the lungs.

These conclusions as to the rôle of the lymph nodes in the pathogenesis of anthrax infection are based mainly on post-mortem and histological investigations, and on counts of bacilli in the tissues at various stages of the disease. An alternative approach is to measure the output of bacilli from infected tissues into the lymph and blood streams, and this has been done in the experiments described in this paper.

METHODS

Pulmonary anthrax

Rabbits were exposed to a passing cloud of single *Bacillus anthracis* spores, using the apparatus described by Henderson (1952). The strain used was M36, which is highly virulent for rabbits. The exposure time was one minute, and the spore concentration varied from 0·9 to 1·5 $\times$ 10^6 spores/litre in different experiments. Assuming a minute volume of 400 ml., 3·6 to 6 $\times$ 10^5 spores would be inhaled; of these, 20–25 per cent would be retained in the lungs (Barnes, 1947). This is sufficient to kill 80–100 per cent of the exposed rabbits, death usually occurring 2 to 5 days after infection.

At various times after exposure, samples of right lymphatic and thoracic duct lymph were collected. The rabbits were anaesthetised with pentobarbitone sodium 32–40 mg./kg. The cervical thoracic duct was cannulated with polyethylene tubing, or with a fine metal cannula attached to such tubing; both were initially filled with sterile heparin solution, 10 mg./ml. If attempts to cannulate the duct failed, lymph was collected from the cut duct by pipette.

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No difference in results between the two methods was found. The flow from the thoracic duct was measured at hourly intervals up to 14 hr. after cannulation ; anaesthesia was maintained during this time.

The right lymphatic duct is difficult to cannulate in the rabbit, since it is a small fragile vessel buried in fat. It was therefore cut and the lymph flowing from it collected by pipette for 15 min. In view of the difficulty in obtaining right duct lymph, in some rabbits the duct was ligated. This we consider diverted the right duct lymph to the thoracic duct. (A description of the operation and the reasons for this conclusion are given elsewhere (Hughes, May and Widdicombe, 1956)). After 2–3 weeks the rabbits were exposed to anthrax and, by subsequent cannulation of the thoracic duct, lymph was collected from the drainage areas of both ducts.

Lymph was plated on tryptic meat agar after suitable serial dilutions, and the plates incubated at 37°. Lymph and blood were plated every 1–2 hr.

Anthrax infection of the fore-foot

Rabbits are so susceptible to the M36 strain of anthrax when it is injected subcutaneously that only a few spores are needed to cause death and the dose cannot be measured accurately. A less virulent strain " Vollum " was therefore chosen, of which the subcutaneous LD_{50} is about 400 spores. The choice of injection site depended on the lymphatic drainage: the middle of the fore-foot was used, between the metacarpals. This site is drained by three or four lymph vessels in the lower limb, which meet and anastomose round the brachial artery before entering the axillary lymph node : this drains into the subclavian duct which is easy to cannulate. In some rabbits, part of the lymph from the foot passes to the pectoral and scapular lymph nodes and then to the subclavian duct. These observations were made in a number of rabbits by injection of Evans Blue solution into the foot followed by post-mortem dissection.

The subclavian lymphatic duct was cannulated with fine polyethylene tubing under pentobarbitone or thiopentone sodium anaesthesia, usually the latter. In survival experiments the rabbit was prevented from tearing out the cannula by passing the tubing behind the *manubrium sterni* and beneath the skin of the left side of the neck to appear at the back of the neck. The rabbit was placed in an open-mesh metal box ($14 \times 4\frac{1}{2} \times 4\frac{1}{2}$ in.)($35{\cdot}6 \times 11{\cdot}4 \times 11{\cdot}4$ cm.) with food and water. During the night the lid of the box was down, so that the rabbit remained in the prone position and its only movements were to readjust its position and to eat and drink. During the day the lid of the box was usually removed so that the rabbit could sit up, but it was not allowed to walk about.

Injection of anthrax spores was made into the foot either immediately after or at various intervals before cannulation of the subclavian duct. The dose was usually about 800 Vollum spores in 0·2 ml., which is about an LD_{80}. At intervals of $\frac{1}{2}$ to 2 hr. the flow rate was recorded and lymph and blood plated (after dilution if necessary) ; this routine was broken at night, when the rabbits were left undisturbed with the lymph draining.

RESULTS

Pulmonary anthrax

In preliminary experiments, rabbits were exposed to a cloud of anthrax spores and at various intervals afterwards samples of lymph were collected for 15 min. from the thoracic and right lymphatic ducts. We tried to do this early in the course of bacillaemia before the terminal stages were present, but owing to the variability of the time course of anthrax in rabbits this was only successful in 12 out of 27 experiments.

With two exceptions the anthrax counts of the blood ranged from 5 to 75 organisms/ml., whereas the lymph samples usually gave counts of several thousand per ml. The abdominal lymph stream was not the source of anthrax bacilli in the cervical thoracic duct, since only in the terminal stages of anthrax did the abdominal lymph give positive cultures. We conclude from these results that, after the stage of mediastinal lymphadenitis, there is a massive exit of

bacilli from the lymph nodes into the thoracic and right lymphatic ducts, and that this occurs in the early stages of bacillaemia when the blood count is relatively low. Thoracic duct lymph contained 250–5,000 organisms/ml.: since the output of lymph is about 3 ml./hr. in the anaesthetised rabbit with pulmonary anthrax, the duct is discharging 750–15,000 organisms/hr. The output of the right lymphatic duct was more difficult to determine since the samples were small and the rates of flow known only approximately. But, assuming that the right lymphatic duct drains at least three-quarters of the lungs and mediastinal lymph nodes (Warren and Drinker, 1942), it is likely that at this stage of the disease up to 60,000 organisms per hour are entering the blood. The blood counts of 5–75 organisms/ml. correspond to a total of 600–10,000 for the whole blood volume.

If the main source of the bacillaemia is bacilli from the lymph it should be possible to prevent or delay this by preventing the infected lymph from entering the blood stream. Using the preparation in which right duct lymph was diverted to the thoracic duct, the latter was cannulated 18–30 hr. after inhalation of spores. Lymph was drained for as long as possible after cannulation (up to 14 hr.) and in this way it was hoped to compare the times of onset of lymph and blood infections. The extreme difficulty of timing the experiments was again apparent. The criterion of success was that both blood and lymph should be sterile at the start of the experiment and that one or both should become infected during its course. This occurred in 3 and partially in 1 out of 18 experiments, the results of which are shown in Table I. In experiments 1, 3 and 4 both blood and lymph were sterile at the start and both became infected during the experiment; the lymph infection preceded the blood infection by 2–4 hr. and in the last 2 experiments the lymph counts were far higher than those in the blood. In experiment 2 the bacillaemia was present initially whereas the lymph was sterile but later became infected. Of the remaining experiments, in 8 both blood and lymph were infected before cannulation and in 6 neither fluid became infected during the experiment.

TABLE I.—*Appearance of Anthrax Bacilli in Blood and Thoracic Duct Lymph after Inhalation of Spores*

Experiment.	Time after exposure (hours).	Lymph count (orgs./ml.).	Blood count (orgs./ml.).
1	26	0	0
	27	*ca.* 150	0
	31	*ca.* 175	*ca.* 100
2	30	0	40
	33	*ca.* 50	15
3	32	0	0
	33	15	0
	35	1,225	40
4	24	0	0
	26	50,000	0
	27	200,000	204

The right lymphatic ducts had been previously ligated. Samples of blood and lymph were cultured at hourly intervals. Selected results are given, corresponding to the last time when both fluids were sterile, the first appearance of bacilli in one fluid, and the presence of bacilli in both.

Anthrax infection in the fore-foot

In our first experiments we examined lymph early in the stage of bacillaemia. The subclavian lymphatic duct was first cannulated and a 15 min. sample obtained. Then, one or two lymphatics in the lower part of the limb were dissected out and cut, and a 15 min. sample of lymph collected.

Fifteen experiments were done, of which 6 showed no lymph infection; the results of the other 9 are given in Table II. In all but 2 experiments there was a greater output of bacilli in the efferent lymph than in the afferent, the difference sometimes being several thousand-fold. Afferent lymph was collected from only one or two out of several lymphatics draining the site of infection so these figures should be multiplied by 2 or 3 to apply to the entire lymph drainage of the foot. During the collection of lymph the node was not massaged (or approached in dissection); on the other hand flexion and extension of the ankle joint inevitably massaged the site of injection, and this may have liberated organisms into the afferent lymph. It is therefore probable that the anthrax counts on afferent lymph are higher than they would be in the quiescent limb.

In the first 6 experiments shown in Table II a large dose, *i.e.*, 8,500 spores, was injected; this was followed by anthrax infection at the site of injection, shown by the presence of oedema and confirmed by culturing the tissue (experiments 5 and 6). In experiments 7, 8 and 9 there was slight or no swelling of the foot and bacilli were discovered in only 2 rabbits by culturing the tissues of the foot; no anthrax bacilli were found in the afferent lymph. The positive cultures from the foot in experiment 8 could be due to the considerable bacillaemia.

TABLE II.—*Living Anthrax Bacilli in Lymph from the Fore-limb after Injection of Spores into the Foot*

Experiment.	Time from injection (hours).	Dose (spores).	Efferent lymph (orgs./hr. $\times 10^{-3}$).	Afferent lymph (orgs./hr. $\times 10^{-3}$).	Axillary lymph node.	Blood (orgs./ml.)	Injection site.
1	24	8,500	1,500	3	Confl.	35	—
2	25	8,500	5	15	+	0	—
3	28	8,500	2,700	1	Confl.	266	—
4	29	8,500	1	4	++	0	—
5	48	8,500	214	0	Confl.	35	Confl.
6	49	8,500	5	0·4	„	1,320	„
7	29	850	6	0	„	0	„
8	46	850	28,300	0	„	Confl.	„
9	47	850	49	0	„	430	0

Efferent lymph was from the cannulated subclavian duct, afferent lymph from two or three lymphatics in the lower limb. Confl. = confluent growth.

In a second series of experiments lymph was collected from the subclavian duct in the *unanaesthetised* rabbit; the experiments usually continued for about 48 hr. Table III gives 4 results in which positive lymph cultures were obtained. Three of the rabbits had outputs of many millions of organisms per hour. Experiment 2 is of particular interest since over 30 million organisms were collected in the subclavian lymph during more than 7 hr. while the blood remained sterile. In experiment 4 the lymph discharged 10,000–30,000 organisms/hr. for nearly 5 hr. while the blood remained sterile. In experiments 2 and 4

the tissues of the foot were oedematous and positive cultures were obtained; this was not seen in experiments 1 and 3.

TABLE III.—*Output of Anthrax Bacilli in Subclavian Lymph in Unanaesthetised Rabbits during Infection of the Fore-foot*

Experiment.	Time from injection (hours).	Duration of samples. Hr.	Min.	Lymph anthrax (orgs./hr. $\times 10^{-6}$).	Blood anthrax (orgs./ml.).
1	24	3	20	1·1	3,000
	27	1	45	6·7	7,500
2	23	18	0	*ca.* 1·0	0
	41	7	0	4·7	0
	48	16	0	7·3	50,000
3	32	0	35	18·2	14,000
4	30	17	0	*ca.* 0·005	0
	47	1	45	0·01	0
	49	3	0	0·03	0

Abridged results from 4 experiments. Average results from several samples are given in each line, with the period over which the samples were collected and time from injection of the beginning of the period. The blood anthrax counts were done at the end of each period.

DISCUSSION

Our results show that before bacillaemia can be recognised the lymph nodes discharge bacilli into the thoracic and right lymphatic ducts; there may be an output of 60,000 organisms per hour while the blood anthrax count is still low. The change from sterile lymph to highly infected lymph is rapid: in three to four hours the counts may rise to 200,000 organisms/ml. Young *et al.* (1946) showed that when guinea-pigs were exposed to a cloud of anthrax spores (the dose rather less than LD_{50}) organisms almost immediately reached the peribronchial lymph nodes and remained there with little change in number for 48 hr. Our rabbits had a considerably higher dose (LD_{80-100}) but the results were similar. Presumably the bacilli multiply in the lymphoid tissue but remain there without appreciable liberation into the blood stream until a critical point is reached and a progressively increasing output of organisms occurs.

The experiments also show that the right lymphatic and thoracic ducts are not the only source of blood bacilli. There might be lymphatic channels opening into the blood stream other than the two main ducts, but dissections suggest that they must be very small. The view that bacilli may enter the blood stream directly from tissues has been put forward by Boquet and Saenz (1931) and by Buchner (1888), but there is no direct support for it. In perfused lungs which had been previously exposed to anthrax spores we found that the blood recirculating through the lung vessels did not become infected. It is most likely that bacilli enter the blood stream directly from the engorged lymph nodes. It is supported by the closeness in time of onset of blood and lymph infections: we may suppose that when the nodes become sufficiently engorged with organisms, bacilli escape both into the efferent lymph and into the blood sinuses.

Unlike the lungs, the tissues of the foot support the multiplication of anthrax bacilli, but the rôle of this in the production of bacillaemia is small in relation to that of the regional lymph nodes. With an injection of about LD_{80} there may be no macroscopic evidence of local infection and there may be no bacilli in a 15 min. sample of afferent lymph. At the same time many millions of bacilli may be escaping from the regional lymph nodes. When larger doses of spores are injected a local lesion is evident, but even when the oedematous foot is massaged there is a far greater number of bacilli in the efferent than in the afferent lymph. The bacilli in the efferent lymph are newly formed in the node, and not passing through from the foot, since the lymph node of the normal rabbit is a highly efficient filter of anthrax bacilli (Widdicombe, Hughes and May, 1955) and inflamed nodes are even more efficient in this respect than normal ones (Smith and Wood, 1949). Multiplication of bacilli in the foot therefore contributes little to the bacillaemia, either because germination of the spores is delayed, or because the foot supports multiplication of bacilli far less readily than the lymph nodes, or because the bacilli are fixed to the tissues and do not appear in the lymph.

In two experiments of this series highly infected subclavian lymph was collected by drainage for several hours while the blood samples remained sterile so that there was no appreciable entry of bacilli into the blood by routes other than the lymph. With pulmonary anthrax, lymph infection preceded bacillaemia by a few hours in three out of four experiments, and cannulation of the appropriate lymph channels only delayed bacillaemia for a short time. Different strains of *B. anthracis* were used in the two types of experiment. With strain M36 in the pulmonary series it is possible that bacilli passed directly into the blood stream from the lymph nodes at about the same time as they entered the efferent lymphatics. With the "Vollum" strain (foot series) the less virulent organisms might have produced a different lymph node response and have entered the lymph stream in preference to the blood sinuses. In addition, the fact that the anthrax counts in lymph draining the mediastinal nodes were considerably smaller than those from subclavian lymph may be due to the different strains of bacilli. Since in both areas involvement of the lymph nodes is an invariable preliminary to bacillaemia and death, it seems highly probable that for reasons unknown the lymphatic system supports the growth of anthrax bacilli more readily than other tissues. Any defensive rôle of the nodes is rapidly eclipsed by the fact that they provide a good environment for multiplication of anthrax bacilli.

SUMMARY

The output of anthrax bacilli from the lymph nodes of infected rabbits has been measured. The animals were infected either by inhalation of anthrax spores or by an injection of spores into the fore-foot. The lymphatics draining the regional lymph nodes were cannulated and the lymph cultured. Several thousand to many million organisms per hour were discharged into the efferent lymph stream in the early stages of bacillaemia.

Cannulation of the efferent lymphatics draining the infected regional lymph nodes sometimes delayed for a few hours the onset of bacillaemia, but did not prevent it.

With anthrax infection of the foot a massive discharge of bacilli from the lymph nodes can occur while few or no organisms are appearing in the lymph draining the primary site of infection; macroscopic evidence of infection in the foot may be absent.

It is probable that anthrax bacilli multiply more readily in the lymph nodes than in other tissues, and that the nodes act as centres for the proliferation and dissemination of anthrax bacilli leading to bacillaemia and death.

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REFERENCES

BARNES, J. M.—(1947) *Brit. J. exp. Path.*, **28**, 385.
BOQUET, A. AND SAENZ, A.—(1931) *C. R. Soc. Biol., Paris*, **107**, 768.
BUCHNER, H.—(1888) *Arch. Hyg., Berl.*, **8**, 217.
CROMARTIE, W. J., BLOOM, W. L. AND WATSON, D. W.—(1947) *J. infect. Dis.*, **80**, 1.
HENDERSON, D. W.—(1952) *J. Hyg., Camb.*, **51**, 372.
HUGHES, R., MAY, A. J. AND WIDDICOMBE, J. G.—(1956) *J. Physiol.*, **132**, 384.
SMITH, R. O. AND WOOD, W. B., Jr.—(1949) *J. exp. Med.*, **90**, 567.
WARREN, M. F. AND DRINKER, C. K.—(1942) *Amer. J. Physiol.*, **136**, 207.
WIDDICOMBE, J. G., HUGHES, R. AND MAY, A. J.—(1955) *Brit. J. exp. Path.*, **36**, 473.
YOUNG, G. A., ZELLE, M. R. AND LINCOLN, R. E.—(1946) *J. infect. Dis.*, **79**, 233.

THE SURVIVAL OF RATS AFTER ADRENALECTOMY

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THE work of Deanesly (1938) and Hill (1951) indicated that the ovary of the rat and mouse elaborated a substance which could prevent the death of such animals after bilateral adrenalectomy. While we were reinvestigating this relation between the ovary and adrenal it became evident that we should have to employ accurate methods of comparison of the times of survival of groups of adrenalectomised rats subjected to different experimental procedures. It was found that certain significant features were overlooked if the usual methods of comparison were used. The object of this paper is to indicate the types of difficulty encountered and the methods employed to overcome them so that accurate comparisons could be made.

We investigated three factors in relation to bilateral adrenalectomy,

(1) Strain differences,
(2) Environmental temperature,
(3) Bilateral öophorectomy,

and analysed our results by a method to be described below.

METHOD

Female rats were used, approximately 6 weeks of age at the time of operation. They were of three strains :

(1) White hybrid rats bred in our own laboratory.
(2) Pure bred Wistar rats from a dealer.
(3) Pure bred Salonika rats obtained by the courtesy of Dr. Baker of Clayton Aniline Co. Ltd., Manchester.

Animals obtained from outside sources were allowed at least one week in our own animal houses to acclimatise pre-operatively. These animal houses were maintained at a thermostatically controlled temperature of 25° throughout the experiments. All animals were fed on cubes of Diet 41 from the Associated London Flour Millers Ltd. The percentage composition of this diet is : Whole meal flour 46, Sussex ground oats 40, fish meal 8, dried skim milk 3, dried yeast 1, cod liver oil equivalent 1, salt 1. Water was unrestricted.

All animals were anaesthetised by intraperitoneal veterinary Nembutal (pentobarbitone sodium) and inhalation of ether. An aseptic technique was used and both adrenals were removed in a one-stage operation through a transverse lumbar incision. The adrenals were removed completely together with the surrounding fat, care being taken not to pierce their capsules. One group of animals for the temperature experiment was kept in an electrically heated chamber at 34–38° checked by thermograph readings. The bilaterally öophorectomised group of animals had this operation at the same time as the adrenalectomy.

Rats surviving more than 8 weeks post-operatively were arbitrarily regarded as indefinite survivors and were killed and examined in an attempt to find adrenal remnants. Tissue from the adrenalectomy site and any fragments suspected of being adrenal were sectioned and examined microscopically to determine their nature.

RESULTS

The results are given in Table I. Animals dying during the first 24 hr. after operation are regarded as having died of post-operative shock rather than of adrenal insufficiency. When these animals and those in which adrenal remnants were found post mortem are excluded, it will be seen that of the animals kept at the normal animal house temperature of 25°, in the series of 16 Salonika rats there were no indefinite survivors, all having died by the 56th day ; of the hybrid rats there was 1 survivor in a series of 31 ; of the Wistar strain 10 survived out of 57. In the group of 38 rats in which öophorectomy was combined with adrenalectomy there were 2 survivors. Of 40 Wistar rats kept at a temperature of 34–38° there were 4 indefinite survivors.

TABLE I.—*Survival of Rats after Bilateral Adrenalectomy*

	Hybrids at 25°	Salonikas at 25°	Wistars at 25°	Wistars at 34–38°	Wistars after öophorectomy
1. Number of rats used	39	22	69	50	43
2. Rats dying within 24 hr. of operation	6	3	1	8	2
3. Rats surviving 56 days with adrenal remnants	2	3	11	2	3
4. Rats surviving 56 days with no adrenal remnants	1	0	10	4	2
5. Rats dying between 24 hr. and 56 days after operation	30	16	47	36	36
6. Total of (4) and (5)	31	16	57	40	38
7. Mean (median) time of death (days)	17·54	13·77	13·85	9·02	8·36
8. Standard deviation (log days)	0·281	0·162	0·350	0·343	0·288

The total in row (6) is the number of animals available for calculation of the mean and standard deviation.

ANALYSIS OF RESULTS

Two difficulties arise in the analysis of these results : (*a*) the mean time of survival and the median (*i.e.*, the time when 50 per cent of the animals have died) do not coincide, and (*b*) there were some animals which lived for 56 days which could not be excluded from the calculations as they had no residual adrenal tissue to account for this prolonged survival. These difficulties can be overcome by the following procedure.

Plotting the number of animals dying each day after adrenalectomy a cocked hat type of curve is obtained (Fig. 1). This curve is asymmetrical with a long tail following the maximum. Altering the abscissa (time) from days to log-days converts the curve into an almost symmetrical "normal" (Gaussian) curve. In this the mean time of survival and the 50 per cent mortality points coincide. The median is a more valid estimate of the effect of treatment than is the mean, but the median cannot be as easily manipulated for statistical comparison as can the mean ; by making the two coincide this difficulty is overcome.

Since some of our animals with no residual adrenal tissue did not die within the arbitrary limit of 56 days after adrenalectomy the right-hand tail of the frequency distribution is indeterminate. Bliss (1937) has devised methods for estimating the means and standard deviations of such truncated distributions. Plotting the number of animals which have died against time sigmoid curves are obtained (Fig. 2). Percentages may be transformed to the corresponding probits

using a suitable table (Fisher and Yates, 1948, Table IX); plotting probits against time (in this case log-days) an almost straight line is obtained (Fig. 3). From the slope of the line a provisional estimate of the standard deviation can be made;

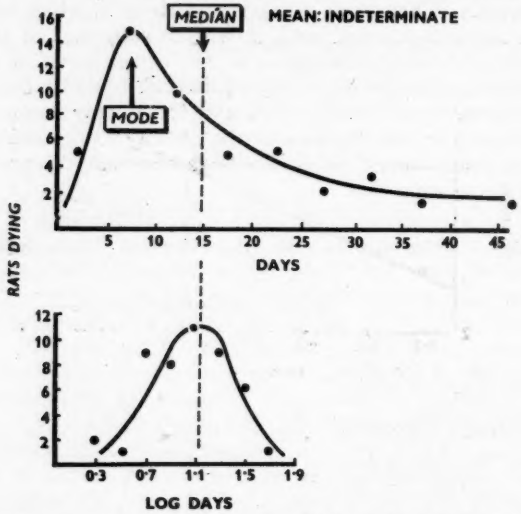

FIG. 1.—Mortality distribution curves for Wistar rats kept at 25° after bilateral adrenalectomy. The ordinates measure the number of rats dying in unit time. Changing the time units from days to log days makes the curve almost symmetrical, the mean, mode and median coinciding.

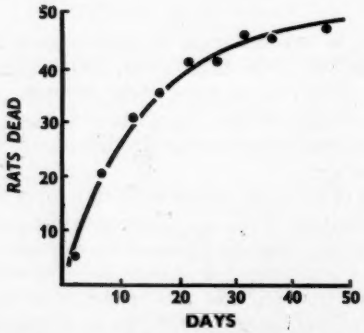

FIG. 2.—Cumulative mortality curve for the same group of rats as in Fig. 1. The ordinates measure the number of rats dead at a given time.

the provisional mean is the intersection of the line with the ordinate for 5 probits; these values together with the point of truncation are then substituted in Bliss' equations from which corrected estimates can be calculated. The process is then repeated using the corrected estimates until repetition produces only a slight change in the estimates of the mean and standard deviation.

It can be seen from Table II that the mean times of death for the 3 strains of rats following adrenalectomy do not differ significantly, nor do the standard deviations of time of death of the Wistar and hybrid strains. The standard deviation of the Salonika rats, however, is significantly smaller than that of the other two strains. These observations are illustrated in Fig. 4, where the number of rats dead at a given time is plotted against time (the curves are drawn

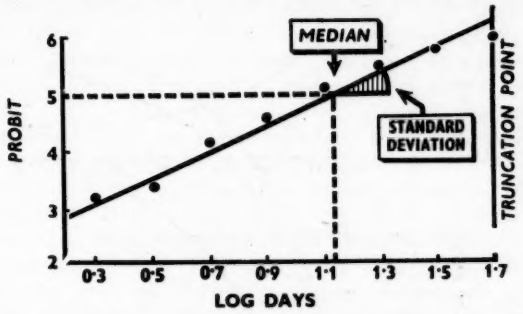

FIG. 3.—Transformed mortality curve for the same group of rats as Fig. 2. The ordinates measure the number of rats dead at a given time in probit units and time has been transformed to log days. The curve is thus converted into a straight line and the standard deviation is given by the abscissal distance separated by one unit of probit. The point of truncation corresponds to 56 days.

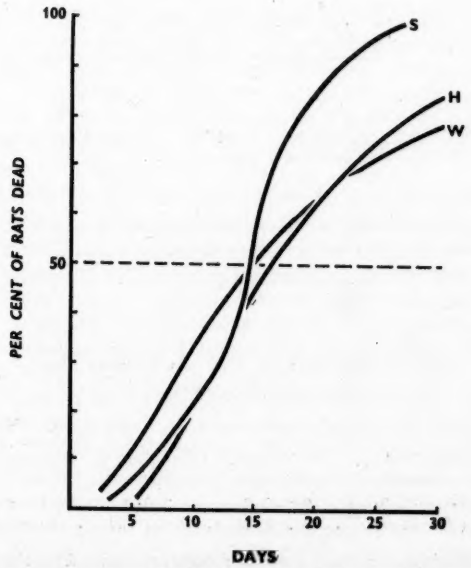

FIG. 4.—The effect of strain differences. S, Salonika, H, Hybrid and W, Wistar rats. Cumulative mortality curves drawn using the parameters calculated from the observations.

using the parameters in Table I). The mean is given by the point in time at which the 50 per cent ordinate cuts the curve. The standard deviation is a measure of the slope of the curve ; the smaller the standard deviation, the greater is the slope. The curves shown for the hybrid and Wistar strains of rats are very similar and lie close together, but that for the Salonika rats rises sharply, corresponding to the smaller standard deviation, and indicates that the deaths are more closely grouped about the mean than for the other two strains.

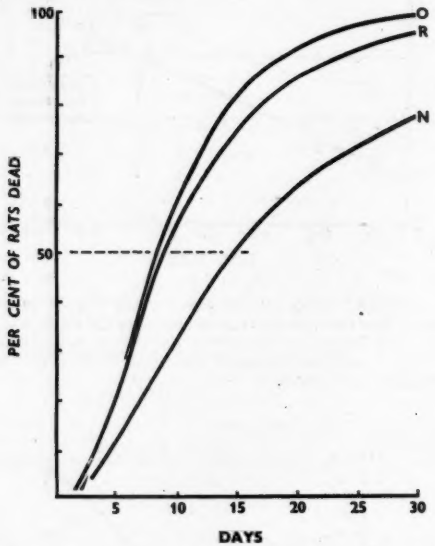

FIG. 5.—N, rats kept at 25° ; R, rats kept at 34–38° ; O, öophorectomised rats. Cumulative mortality curves drawn using calculated parameters.

It also follows from Table II that both öophorectomy and raising the environmental temperature of rats after adrenalectomy significantly reduce the mean time of survival, but do not significantly alter the standard deviation. This is illustrated in Fig. 5.

TABLE II.—*The Probability,* P, *that the Groups Compared are Drawn from the Same Population*

	Hybrids *v.* Salonikas	Wistars *v.* Hybrids	Salonikas *v.* Wistars	Wistars at 25° *v.* Wistars at 34–38°	Wistars *v.* öophorectomised Wistars.
Mean	0·2–0·1	0·3–0·2	>0·9	*0·01–0·001	*0·01–0·001
Standard deviation	*0·02–0·01	0·2–0·1	*<0·001	0·9–0·8	0·2–0·1

If $P < 0·05$, it is considered unlikely that the groups are drawn from the same population. Such significant differences are starred.

Except for the rats in column (4), the animals were all kept at 25°.

DISCUSSION

When the time-mortality curves of two experiments are very different comparison by calculation of statistics is not important, but when the curves are very similar important differences may be missed if appropriate methods of analysis are not used. Some of the most careful workers have used the mean time of survival when comparing the effects of various glandular extracts in adrenalectomised animals (Firor and Grollman, 1933; Cartland and Kuizenga, 1936; Bodansky, Agress, Campbell and Herrmann, 1936). The mean is unsatisfactory because it does not measure a constant level of effect unless the distribution is "normal" (Bliss, 1937). The median is a satisfactory estimate of effect but cannot be manipulated with ease mathematically for the purpose of comparison. It is thus desirable if possible to transform time so that the distribution is "normal" in which case the mean and the median coincide. The simple transformation of time from days to log-days, as used here, is often adequate and is applicable, for example, to the mortality of rats after adrenalectomy (Gillman and Golberg, 1942) and also to that of guinea-pigs (Simmons and Whitehead, 1936).

Previous workers have attempted to explain prolonged survivals in their series. Remnants of adrenal left at operation, or accessory adrenal tissue have been postulated and reported at autopsy, frequently without confirmation by microscopy. We have found macroscopic post mortem appearances misleading; histological study has shown several of our macroscopic "positives" to be lymph nodes. After careful and detailed microscopy we have been unable to find either remnants of adrenal tissue left at operation or ectopic adrenal in 17 of 38 rats which survived beyond 56 days.

A small "tail" of prolonged survivors does not invalidate the expression of results by the usual methods of mean and standard deviation. Several workers however, as for example Carr (1931), Bodansky *et al.* (1936) and Swann (1937), use this method of expressing their results in time-mortality terms, but ignore an appreciable number of prolonged survivors, and their findings cannot therefore be accepted. By using the method of Bliss (1937) prolonged survivors are taken into account, and this difficulty overcome.

Contrary to the findings of Carr (1931), but in agreement with Cowie (1949) and Snyder and Wyman (1951), our results show that öophorectomy combined with adrenalectomy shortens the survival period. This suggests that the ovary may assume some of the functions of the adrenal, a hypothesis which we are studying further by histological examination of the ovaries of adrenalectomised rats. Earlier work suggests that the hormone of the corpus luteum may ameliorate the effects of adrenalectomy (Gaunt and Hays, 1938; Greene, Wells and Ivy, 1939).

It is interesting that while the median survival time of the Salonika rats does not differ significantly from that of the other strains the standard deviation is significantly smaller. This indicates that the deaths are more closely grouped about the median in the Salonika rats and explains the absence of an indeterminate tail of prolonged survivors. Such strains with small standard deviations are useful when a sharp end-point is needed in experiments using time-mortality observations.

In the present experiments we have not attempted a detailed investigation of either the factors which may determine different survival times in different

strains of rats, or those involved when the environmental temperature is varied. With our findings, however, it is now possible to follow Fisher's (1945) methods and design " factorial " experiments for this purpose and for the study of ovarian changes after adrenalectomy.

SUMMARY

Two difficulties arise in comparing the survival times of bilaterally adrenalectomised rats under varying experimental conditions. The median and mean survival times do not coincide ; this can be adjusted by using a logarithmic scale for time. There is often a number of rats surviving beyond the time at which it is convenient to terminate the experiment ; a correction may be made for this by a method described by Bliss.

The 3 strains employed in this experiment showed no significant difference in median survival time but deaths in one strain (Salonika) were more closely grouped about the median. Raising the environmental temperature and öophorectomy reduced the median survival time significantly.

REFERENCES

Bliss, C. I.—(1937) *Ann. appl. Biol.*, **24**, 815.
Bodansky, M., Agress, C. M., Campbell, K. R. and Herrmann, C. L. —(1936) *Endocrinology*, **20**, 684.
Carr, J. L.—(1931) *Proc. Soc. exp. Biol., N.Y.*, **29**, 128.
Cartland, G. F. and Kuizenga, M. H.—(1936) *Amer. J. Physiol.*, **117**, 678.
Cowie, A. T.—(1949) *J. Endocrin.*, **6**, 94.
Deanesly, R.—(1938) *Proc. roy. Soc. B.*, **126**, 122.
Firor, W. M. and Grollman, A.—(1933) *Amer. J. Physiol.*, **103**, 686.
Fisher, R. A.—(1945) ' The design of experiments.' 4th ed. Edinburgh (Oliver and Boyd).
Idem and Yates, F.—(1948) ' Statistical tables for biological, agricultural and medical research.' 3rd ed. Edinburgh (Oliver and Boyd).
Gaunt, R. and Hays, H. W.—(1938) *Amer. J. Physiol.*, **124**, 767.
Gilman, J. and Golberg, L.—(1942) *Endocrinology*, **31**, 201.
Greene, R. R., Wells, J. A. and Ivy, A. C.—(1939) *Proc. Soc. exp. Biol., N.Y.*, **40**, 83.
Hill, R. T.—(1951) *Colloq. int. Cent. nat. Rech. sci.*, **31**, 452.
Simmons, H. T. and Whitehead, R.—(1936) *J. Physiol.*, **88**, 235.
Snyder, J. G. and Wyman, L. C.—(1951) *J. Endocrin.*, **49**, 205.
Swann, H. G.—(1937) *Amer. J. Physiol.*, **118**, 798.

THE EFFECT OF COBALT CHLORIDE AND SYNTHALINE A ON REDUCED GLUTATHIONE IN BLOOD AND TISSUES IN RATS WITH PARTIAL PANCREATIC DUCT LIGATION

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THE administration of alloxan causes in some animals a drop in reduced glutathione in the blood and/or certain tissues (Eckert, Kalina and Pillemer (cited by Houssay, 1950); de Caro and Rovida, 1937; Leech and Bailey, 1945; Brückmann and Wertheimer, 1947; Binet, Wellers and Marquis, 1949; Bhattacharya, Robson and Stewart, 1956; Hultquist, 1955, 1956*a*, *b*). In the atrophied remains of the pancreas on the lienal side of a duct ligation, reduced glutathione is not affected by an alloxan injection (Hultquist, 1955, 1956*a*, *b*). Cobalt chloride which, like alloxan, affects the islets of Langerhans but damages the α-cells instead of the β-cells, might conceivably affect the level of reduced glutathione. Ling and Chow (1953, 1954*a*, *b*) and Register (1954) found that vitamin B_{12} deficiency in experimental animals causes a decrease in reduced glutathione and that administration of vitamin B_{12} raises the value in animals suffering from vitamin deficiency. Ling and Chow (1953) administered cobalt chloride for three weeks to rats with vitamin B_{12} deficiency, but they noted no rise in the low SH level in the blood.

The object of the present investigation was to study the occurrence of reduced glutathione in rats, in the blood and tissues, and in the pancreas remains left after partial duct ligation, after the injection of a dose of cobalt chloride sufficient to produce damage to the α-cells in the islets of Langerhans. The experiments were also extended to include synthaline A, which, like cobalt chloride, damages the α-cells. Davis (1952) could not observe any definite change in the blood glutathione during the first few hours after a synthaline injection.

METHODS AND MATERIAL

Litter-mates white male rats were used for the experiments. In a few groups of animals, ligation of the ducts to the lienal one-fourth to one-third of the pancreas (partial duct ligation) was carried out at the age of five months. This form of ligation does not seem to have any noticeable effect on the exocrinic pancreas secretion or on the animals' growth and weight increase. It permits, however, determination of the glutathione in the atrophied pancreas, which consists of dilated tubules, islet tissue and connective tissue, and can thus provide observations reflecting in some measure the changes which take place in the glutathione values in the islet tissue. Rats subjected to the same surgical intervention except for ligation were used as controls. When the animals were seven months old the amount of reduced glutathione in the blood, liver, kidneys, pancreas and the atrophied lienal part of the pancreas was determined. Some animals were given a subcutaneous injection of 0·5 per cent cobalt chloride ($CoCl_2$), 50 mg. per kg. body weight, or 0·25 per cent synthaline A, 4 mg. per kg. body weight. At different times after the injection the animals were killed and the organs freed as far as possible from blood. After homogenisation of the tissues in ice-cold

2 per cent metaphosphoric acid, the reduced glutathione was determined both spectrophotometrically at 525 mμ by the *Thompson* and *Watson* (1952) method, with the sodium nitroprusside reaction (N), and by an iodimetric method (I) according to which the ascorbic acid is first titrated with dichlorphenolindophenol.

RESULTS

Twelve hours after the administration of cobalt chloride there was observed a significant rise in reduced glutathione in the liver and pancreas and a probable rise in the atrophied lienal part of the pancreas resulting from the ligation (Table I). The blood, which showed a probable rise 15 min. after the injection, displayed no change 12 hr. later, and the kidneys showed a tendency towards a decrease, although not consistently so, and not to any marked degree. Control animals showed approximately similar conditions (Table II).

The rise in the glutathione level in the liver, and in both the remaining normal duodenal pancreas and the lienal atrophied pancreas, still persisted 48 hr. after the administration of cobalt, the values being 329 (I) and 273 (N) for the liver, 88 (N) for the duodenal part of the pancreas and 68 (N) for the lienal pancreas.

Synthaline A caused an increase in the ascorbic acid and glutathione levels in the liver after 15 min., but the glutathione increase was of a lower degree and apparently of a more transient nature than that observed after the cobalt chloride. On the other hand, synthaline A gave a significant decrease in both the ascorbic acid and glutathione levels in the kidneys after 12 hr. In microscopic sections of the kidneys, considerable degenerative changes were noted in the tubules.

The haematocrit values for partially ligated animals which had received a cobalt chloride injection 15 min. before they were killed did not diverge to any great extent from the values in the control animals (55 and 54). On the other hand, 12 hr. after the cobalt chloride injection, the haematocrit values rose to 63 as compared with 55. The same conditions were found in control animals 12 hr. after injection (65 as compared with 57). Twelve hours after a synthaline A injection, on the other hand, no effect on the haematocrit values was noted (54 as compared with 55 among partially ligated animals, and 55 as compared with 57 for control animals).

DISCUSSION

In a dose sufficient to produce α-cell damage in the islets of Langerhans, cobalt chloride causes, according to our observations, a considerable increase in reduced glutathione in certain organs, and synthaline A has a similar effect. The rise following administration of cobalt chloride takes place fairly slowly and persists for a considerable time after the injection. The immediate rise in the blood is not accompanied by an increase in the haematocrit values, but the increase observed later may have some connection with the haematocrit rise. The possibility that the glutathione increase is due to the fact that the organs in the cobalt-injected animals contain larger quantities of red blood cells would seem to be very unlikely, especially in view of the technique, and in the synthaline-injected animals this possibility is out of the question.

Thus the results differ from those obtained by Ling and Chow (1953) after administration of cobalt chloride to rats with vitamin B_{12} deficiency. We have

TABLE I.—*Reduced Glutathione Content (mg./100g.) after Injection of Cobalt Chloride in Rats with Partial Pancreatic Duct Ligation.*

TABLE I.—*Reduced Glutathione Content (mg./100g.) after Injection of Cobalt Chloride in Rats with Partial Pancreatic Duct Ligation.*

	Blood.		Liver.			Pancreas. Duodenal part.			Pancreas. Lienal part.	Kidney.		
	I.	N.	Asc.	I.	N.	Asc.	I.	N.	N.	Asc.	I.	N.
Controls	46	36	24	232	192	10	123	58	47	15	227	104
$CoCl_2$, 15 min. after injection	60	48	24	284	236	9	134	45	46	14	205	91
d.f.	4	6	9	9	9	7	7	10	7	10	10	10
t	3·46*	1·44	0·278	3·72†	2·88*	0·634	0·84	1·86	0·292	0·274	2·33*	1·82
$CoCl_2$, 12 hr. after injection	46	38	31	388	317	8	209	82	56	11	233	97
d.f.	5	8	13	13	13	10	10	13	10	12	12	12
t	—.	0·349	5·06‡	17·5‡	13·0‡	1·83	4·57‡	4·29‡	2·92*	2·40*	0·915	3·41†
Synthaline A, 15 min. after injection	46	40	31	263	221	10	138	51	52	14	218	95
d.f.	3	5	10	10	10	7	7	9	7	10	10	10
t	—	0·391	15·8‡	2·56*	2·40*	—	0·69	1·09	1·08	0·218	0·89	1·30
Synthaline A, 12 hr. after injection	49	42	24	254	169	8	153	61	45	8	116	53
d.f.	8	11	14	14	14	10	10	14	12	14	13	13
t	0·66	1·33	—	2·46*	1·95	2·39*	1·80	0·537	0·553	4·85‡	13·8‡	8·95‡

I = iodimetric method. N = nitroprusside method. Asc. = ascorbic acid. d.f. = degrees of freedom. * = probable † = very probable. ‡ = significant.

TABLE II.—*Reduced Glutathione Content (mg./100g.) after Injection of Cobalt Chloride in Sham-operated Rats.*

	Blood.		Liver.			Pancreas.			Kidney.		
	I.	N.	Asc.	I.	N.	Asc.	I.	N.	Asc.	I.	N.
Controls	43	39	26	236	205	8	136	59	15	218	103
$CoCl_2$, 12 hr. after injection	52	49	31	375	321	8	213	85	12	231	107
d.f.	7	6	7	8	8	—	8	8	6	7	7
t	1·45	2·32	5·52‡	16·7‡	15·2‡	—	6·11‡	3·07*	1·64	0·89	0·66
Synthaline A, 12 hr. after injection	53	49	27	252	188	9	164	69	8	134	68
d.f.	4	4	5	6	6	5	6	6	5	6	6
t	1·97	2·77*	0·32	1·16	2·98*	0·87	3·06*	1·38	14·7‡	16·3‡	4·72†

I = iodimetric method. N = nitroprusside method. Asc. = ascorbic acid. d.f. = degrees of freedom. * = probable. † = very probable. ‡ = significant.

not been able to find any explanation of our observations with regard to cobalt chloride and synthaline A, but for cobalt chloride, at least, the explanation may possibly be sought along the same lines as the reasoning advanced by Dubnoff (cited by Register, 1954) to account for the effect of vitamin B_{12} on glutathione, namely, that it reduces disulphide linkages.

Lazarow (1954) and Patterson and Lazarow (1955) observed that cobalt, as well as zinc and ferrous iron, when given intravenously before an injection of alloxan, protected against diabetes. The effect was not obtained if the metals were administered 15 min. after the adminstration of alloxan. Cobalt was five times as effective as zinc. These investigators assumed that the metals " may combine with alloxan or catalyse its destruction, thereby lowering the effective concentration of alloxan in the β-cell, or they may reversibly combine with the sulfhydryl groups that react with alloxan and thereby afford an indirect protection ". The results of my experiments would seem to indicate that the cobalt effect observed by Lazarow and Patterson is connected with the rise in the glutathione level in, for instance, the blood and liver which is already clearly observable 15 min. after the administration of cobalt. As the effect of a cobalt administration is only observed if the cobalt is given before an alloxan injection, it would seem improbable that the effect is due to destruction of α-cells.

SUMMARY

The injection of cobalt chloride is followed in rats by an increase in reduced glutathione in the liver and pancreas and in the part of the pancreas atrophied after a partial duct ligation. In the kidneys there is a slight decrease. Synthaline A produces a slight increase in the liver glutathione and a considerable decrease in the kidney glutathione, the latter reaction being accompanied by severe damage to the kidney.

REFERENCES

Bhattacharya, S. K., Robson, J. S. and Stewart, C.P.—(1956) *Biochem. J.*, **62**, 12.
Binet, L., Wellers, G. and Marquis, M.—(1949) *C.R. Acad. Sci., Paris*, **229**, 1185.
Brückmann, G. and Wertheimer, E.—(1947) *J. biol. Chem.*, **168**, 241.
Davis, J. C.—(1952) *J. Path. Bact.*, **64**, 575.
de Caro, L. and Rovida, E.—(1937) *Boll. Soc. ital. Biol. sper.*, **12**, 609.
Houssay, B. A.—(1950) *Amer. J. med. Sci.*, **219**, 353.
Hultquist, G. T.—(1955) *Nord. med.*, **53**, 975.—(1956*a*) *Ibid.*, in press.—(1956*b*) *Acta endocr., Copenhagen*, in press.
Lazarow, A.—(1954) Proc. Symp. Ridgefield, Connecticut, Nov. 1953. New York (Acad. Press), p. 49.
Leech, R. S. and Bailey, C. C.—(1945) *J. biol. Chem.*, **157**, 525.
Ling, C. T. and Chow, B. F.—(1953) *Ibid.*, **202**, 445.—(1954*a*) *Fed. Proc.*, **13**, 253.—(1954*b*) *J. biol. Chem.*, **206**, 797.
Patterson, J. W. and Lazarow, A.—(1955) ' Determination of glutathione. Methods of biochemical analysis II.' New York (Interscience Publ. Ltd.), p. 259.
Register, U. D.—(1954) *J. biol. Chem.*, **206**, 705.
Thompson, R. H. S. and Watson, D.—(1952) *J. clin. Path.*, **5**, 25.

THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*. VIII: FRACTIONATION OF THE INTRACELLULAR MATERIAL OF *BACILLUS ANTHRACIS*

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SMITH, Keppie and Stanley (1953*a*) separated capsulated *Bacillus anthracis* from the body fluids of guinea-pigs dying of anthrax in sufficient quantity and purity for chemical analysis. The organisms were almost completely dissolved by two mild methods (Smith, Keppie and Stanley 1953*b*); the fractionation of this material is now described. It is important to note that the crude material is a mixture of capsular and somatic substances and numerous attempts to remove the capsule preferentially from the organism have so far failed (Smith *et al.*, 1953*b*).

The intracellular material of *B. anthracis* resembles the extracellular substances produced *in vivo* in having anti-phagocytic and virulence-enhancing activities (Keppie, Smith and Harris-Smith, 1953), but it differs from them in two important respects. It does not actively immunise animals against infection (Keppie *et al.*, 1953), and it does not contain the anthrax toxin (Smith, Keppie and Stanley, 1955). Any toxic and immunising activities were not destroyed by the method of extraction used because similar treatment of the extracellular products did not affect these activities (Keppie *et al.*, 1953; Smith *et al.*, 1955). Thus, in the fractionation of the mixture of somatic and capsular material of *B. anthracis* grown *in vivo* we are concerned solely with aggressive action, *i.e.*, with ability to interfere with various host defence mechanisms such as phagocytosis.

All previous chemical work on *B. anthracis* has been carried out with organisms grown *in vitro* and has been concerned only with the isolation of one or other of the two established constituents of the organism, *i.e.*, poly-D-glutamic acid or the polysaccharide (Ivanovics and Bruckner, 1937; Ivanovics, 1940). The methods of isolation were relatively drastic. Here the intracellular material has been separated by a mild treatment into its main constituents for subsequent biological assay.

MATERIALS AND METHODS

Ammonium carbonate extract of B. anthracis (*N.P. strain*) *grown* in vivo.—Prepared as described by Smith *et al.* (1953*a*, *b*). The ammonium carbonate, which represented approx. 50 per cent of the total solids in solution, was lost during freeze-drying and constituted only approx. 2 per cent of the freeze-dried solid.

"*Ballotini*" *extract of* B. anthracis (*N.P. strain*) *grown* in vivo.—Prepared as described by Smith *et al.* (1953*a*, *b*). This extract and the ammonium carbonate extract contained the salts of the Locke's solution which were left in the bacterial sludge after the final washing of the latter.

"*Ballotini*" *extracts of* B. anthracis (*strains N.P. and* "*Sterne*") *grown* in vitro.—*B. anthracis* was grown in tryptic meat broth shaken at 37° for 16 hr. The organisms (0·4 g. from

250 ml. culture) were collected by centrifugation, washed with ice-cold Locke's solution and extracted by shaking with " ballotini " as described by Smith *et al.* (1953*a*).

Assays for immunising, anti-phagocytic and virulence-enhancing activity.—See Smith and Gallop (1956). Activities are quoted as the lowest concentration of a material having the stipulated effect.

Anti-complementary activity.—R.B.C. from defibrinated sheep blood (6 per cent v/v in saline) were sensitised at 37° for 10 min. with excess haemolytic antibody (5 M.H.D.). Serial dilutions in saline of the material to be tested (8 drops) were incubated at 37° for 1 hr. with a quantity of guinea-pig complement which was just sufficient to produce complete lysis in control tubes. The suspension of sensitised cells (5 drops) was added and the mixture incubated at 37° for 1 hr. and left at 0° overnight. Activities of samples are quoted as the lowest concentration which inhibited the lysis of at least half of the added R.B.C. This assay detects 2-fold differences in anti-complementary activity.

Analytical methods

Methods for nitrogen, P, carbohydrate, hexosamine, absorption of ultraviolet light and glutamic acid were as used by Smith and Gallop (1956).

Amino N.—The colourimetric method of Frame, Russel and Wilhelmi (1943).

Pentose.—By the method of Markham and Smith (1949).

" *Free lipid* ".—The material extracted from the dry solid by ether.

" *Combined lipid*".—Determined as described by Smith and Gallop (1956) under " lipid ".

Polyglutamic acid.—The sample (2 mg.) was placed on strips of filter paper and separated by the paper electrophoretic method of Flynn and de Mayo (1951) using a potential gradient of approx. 6 volts per cm. at 20° for 5 hr. Polyglutamic acid which migrated rapidly towards the anode was detected by the method of Strange and Harkness (1953). The coloured area in test samples was compared visually with that of similarly treated standard samples of purified polyglutamic acid.

Paper chromatography.—By the methods of Partridge (1948 ; 1949) for sugars and of Consden, Gordon and Martin (1944) for amino acids.

EXPERIMENTAL AND RESULTS

Fractionation of the Ammonium Carbonate Extract of B. Anthracis *Grown* in vivo.

The ammonium carbonate and " ballotini " extracts of the organisms from infected guinea-pigs had similar anti-phagocytic (0·5 per cent), anti-complementary (0·25 per cent) and virulence-enhancing (5 per cent) activity. The ammonium carbonate extract was the easier to prepare and was therefore used in the preparation of the following materials. All operations were carried out at 0° and pH 7.

(1) *Diffusate.*—Bacterial extract (1 g.) in water (20 ml.) was dialysed in a Cellophane sac against three changes of water (200 ml.). The combined diffusates were freeze-dried.

(2) *Protein A.*—The dialysate from (1) was diluted to 32 ml. and barium acetate (0·32 g.) added. After 1 hr., the precipitate was collected by centrifugation, and washed with 1 per cent barium acetate solution (5 ml.). It was suspended in water (7 ml.) and the suspension shaken gently with the sodium form of Zeocarb 215 (The Permutit Co. Ltd., approx. 1 g. of moist granules) for 30 min. After standing overnight, the Ba^{++}-free solution was pipetted off from the ion-exchange resin and dialysed against distilled water. Because this fraction was rendered partially insoluble by freeze-drying, it was retained in solution for biological testing and further fractionation. A small amount was freeze-dried for chemical analysis.

(3) *Crude polyglutamic acid.*—Ethanol was added to the supernatant solution from (2) to a concentration of 5 per cent (v/v). After 16 hr., the sticky precipitate was collected and mixed with water (5 ml.) and the sodium form of Zeocarb 215 (approx. 2 g. moist granules). The mixture was shaken for 30 min. and then the Ba^{++}-free solution was separated from the resin, dialysed and freeze-dried.

(4) *Protein B.*—Ethanol was added to the supernatant solution from (3) to a final concentration of 33 per cent (v/v). After 16 hr. the precipitate was collected, washed with a mixture of 1 per cent barium acetate solution (2 vol.) and ethanol (1 vol.). The precipitate was then mixed with water (10 ml.) and the sodium form of Zeocarb 215 (approx. 1 g. of moist granules) and the mixture shaken for 30 min. After removing the resin the Ba^{++}-free solution was dialysed and freeze-dried.

(5) *Crude polysaccharide.*—The supernatant from (4) was dialysed and freeze-dried.

TABLE I.—*Yields, Analysis and Aggressin Activity of Fractions from the Intracellular Material of* B. anthracis

(All values per cent)

Fraction.	Yield.	N.	Carbohydrate (as galactose).	Glutamic acid.	Polyglutamic acid.	Anti-phagocytic activity (lowest active conc.).	Anti-complementary activity (lowest active conc.).
Diffusate . .	45	4·4	8·5	1	—	2·0	Inactive at 1·0
Protein A . .	8	13·7	2·0	19	1	0·1–0·05	0·16
Crude polyglutamic acid . .	8	10·0	2·0	82	75	0·3	0·4
Protein B . .	14	15·1	3·6	20	2	0·1–0·05	0·24
Crude polysaccharide . .	9	7·6	30·0	4	—	Inactive at 2·0	Inactive at 1·0

Table I shows the yields, analyses and aggressive activity of the fractions described above; all figures are the average of a number of similar experiments. The success of the fractionation method in separating the polyglutamic acid, the polysaccharide and the proteins is indicated by the analyses.

The diffusate had P, 1·3; hexosamine, *nil*, and ash 63 per cent. Paper chromatography after hydrolysis with 0·5 N-HCl at 100° for 1 hr. showed the presence of glucose and ribose; after hydrolysis with 3 N-HCl at 100° for 16 hr. glycine, alanine, aspartic acid and glutamic acid were detected. The ultra-violet absorption spectrum of a solution had a maximum at 260 mμ. The anti-phagocytic activity of this fraction was non-specific and was caused by the hypertonicity of solutions. Identical anti-phagocytic activity was shown by solutions of NaCl which had a similar hypertonicity. The diffusate had no virulence-enhancing activity at 2 per cent.

Polyglutamic acid.—The crude polyglutamic acid had appreciable anti-phagocytic activity. The purification of polyglutamic acid from this fraction, its criteria of purity and its biological and chemical properties are given in detail by Zwartouw and Smith (1956). Only relevant facts are reported here. Removal of active protein impurities reduced the anti-phagocytic activity, and the final purified product, which contained less than 0·2 per cent of foreign amino acids, was active at 0·8 per cent. It was active in the virulence-enhancing test at 2 per cent and had no anti-complementary activity at 1 per cent.

The polysaccharide was purified (see Smith and Zwartouw, 1956) and had no anti-phagocytic, virulence-enhancing or anti-complementary activity at 1 per cent.

The Protein Fractions of B. anthracis *Grown* in vivo.

Proteins A and B had the highest aggressive activity of the fractions from *B. anthracis*. The remainder of this paper is concerned with their chemistry and with the relevance of their biological activity to the aggressive action of intact *B. anthracis in vivo*. Both proteins were inactive as immunising antigens at 0·7 per cent and showed no marked activity in the virulence-enhancing test at 1 per cent. Relatively high concentrations of some batches of Protein A had a lethal effect on *B. anthracis* (strain N.P.). This peculiar effect was destroyed by incubating a sterile solution of the protein at 37° overnight, a procedure which did not alter the anti-phagocytic or anti-complementary activity.

The analysis of proteins A and B is given in Table II. Paper chromatography of their acid hydrolysates showed that the following amino acids were present in both fractions: glycine, alanine, phenylalanine, valine and/or methionine, leucine and/or iso-leucine, threonine, serine, cysteine, proline, aspartic acid, glutamic acid, lysine, histidine and arginine. Their ultracentrifugal and electrophoretic behaviour at pH 8 showed them to be heterogeneous.

TABLE II.—*Analyses of Proteins A and B from* B. anthracis *Grown* "in vivo"

	Protein A (%).	Protein B (%).
Nitrogen	13·7	15·1
Phosphorus	0·25	0·81
Carbohydrate (as galactose)	2·0	3·6
(as ribose)	1	2
Pentose	1	2
Glutamic acid	19	20
Polyglutamic acid	1	2
Free lipid	*Nil*	*Nil*
Combined lipid	5·7	1·6
Combined lipid phosphorus	0·02	*Mil*
Absorption of ultraviolet light	No max. at 260 mμ	Max. at 260 mμ

Exhaustive attempts were made to purify the aggressin in proteins A and B without success.

Attempts at subfractionation.—Fractionation with ammonium sulphate, with ethanol at 0°, with barium acetate (1 per cent) and ethanol at 0°, and by electrophoresis in the Tiselius apparatus and on paper gave subfractions which all had similar activity. The active material was not preferentially adsorbed on ion exchange resins, Fuller's earth powder and precipitates of calcium phosphate or benzoic acid. Paper chromatography with 20 per cent (w/v) NaCl as solvent did not separate the active material.

Removal of protein by the Sevag method.—Solutions of proteins A and protein B (1 per cent) containing NaCl (6 per cent) were shaken with a half volume of chloroform-butanol mixture (4 : 1). The precipitated gel was removed and the process repeated until no further gel formed. The deproteinised solutions (0·1 per cent) which contained most of the original P and pentose, were inactive in the anti-phagocytic test.

Enzymic degradation.—Trypsin at pH 8 and ficin at pH 7 produced considerable hydrolysis of both proteins but no significant loss of anti-phagocytic activity.

Some concentration of active material was effected by dialysis after treatment with trypsin.

A 1 per cent solution of mixed protein A and B containing a little $CHCl_3$ was treated with crystalline trypsin (0·01 per cent) and maintained at pH 8 and 20° for 24 hr. A further quantity (0·01 per cent) of trypsin was added and after a second 24 hr. the mixture was divided into two parts. One was ultrafiltered through a collodion sac almost to dryness and the ultraconcentrate and ultrafiltrate reserved. The other part was dialysed against water, the dialysate reserved and the diffusate recovered by freeze-drying. Table III summarises the results of biological assays on these fractions. It will be noted that although the anti-phagocytic activity was not affected by treatment with trypsin the anti-complementary activity was destroyed.

TABLE III.—*Action of Trypsin on a Mixture of Proteins A and B*

Sample.	Amino N as per cent total N.	Yield per cent.	Anti-phagocytic activity (lowest active conc. per cent).	Anti-complementary activity (lowest active conc. per cent).
(1) Untreated protein	11	—	0·1	0·1
(2) Trypsin control*	—	—	Inactive	Inactive
(3) Protein control*	17	—	0·1	0·2
(4) Protein + trypsin*	40	—	0·1	Inactive at 1·0
Fractions from (4)				
Ultraconcentrate	23	43	0·05	Inactive at 1·0
Ultrafiltrate	51	48	Inactive at 0·1	Inactive at 1·0
Dialysate	16	39	0·05	—
Diffusate	52	47	Inactive at 0·1	—

* The samples were kept for 48 hr. at pH 8 and 20°.

An Increase of Phagocytosis Resistance in B. anthracis *after Treatment with Proteins A and B.*

In view of the failure of all attempts to fractionate the anti-phagocytic material, attention was given to the behaviour of proteins A and B in the anti-phagocytic test. It was possible that these mixtures of cellular constituents were not true aggressins which directly interfered with phagocytosis but merely appeared to have aggressive activity by virtue of a nutritional effect on the growing test organism (*B. anthracis* strain N.P.). The nutritional effect might operate in two ways, by stimulating growth so that more organisms were present to be phagocytosed or by inducing the production by the test organism of anti-phagocytic substances or mechanisms. Such explanations could account for the failure to fractionate the protein mixtures (obtained from *B. anthracis* strain N.P.) because a number of different constituents might have so acted. Therefore, an analysis was made of the mode of action of proteins A and B in the routine assay for anti-phagocytic activity. Details of this test, which is based on the viable count of organisms surviving phagocytosis, are given by Keppie *et al.* (1953); the numbers of organisms counted in the tests were such that statistical analysis has shown that a difference of 10–15 in the percentage of organisms

surviving phagocytosis in experimental and control samples was significant (Smith and Gallop, 1956).

Effect on growth

Proteins A and B did not cause the numbers of the test organisms to increase in the routine anti-phagocytic assay. The test was made without the phagocytes, *i.e.*, using plasma (0·2 ml.) instead of blood (0·4 ml.). After 1½ hr. at 37° the bacterial count in tubes containing a mixture of proteins A and B (0·5 per cent) or their hydrolysate (3 N-HCl, 100°, 16 hr.) was the same as that in control tubes.

Increased resistance to phagocytosis by treatment with proteins A and B at 37°.

Anthrax bacilli were tested for susceptibility to phagocytosis after the following treatment. The organisms were held for ¾ hr. in a system comparable to the usual test but containing plasma (0·2 ml.) instead of blood ; the experimental sample contained a mixture of proteins A and B (0·5 per cent). The mixtures were then centrifuged and the supernatants removed. Half the deposit of organisms was reserved and the other half was washed twice at 0° with gelatin-Locke solution (2 ml.). The unwashed and washed pre-treated organisms were then used in the phagocytosis test but no further aggressin was added and the mixtures were only incubated ¾ hr. In twelve experiments of this kind it was found that the pre-treated organisms had acquired resistance to phagocytosis. Thus, the average percentage of organisms remaining unphagocytosed among the washed and unwashed bacilli was respectively 14 and 19 in the controls but 30 and 44 after pre-treatment with bacterial proteins.

Increased resistance to phagocytosis by treatment with proteins A and B at 0°.

Experiments were made to test whether the increased resistance to phagocytosis of the test organism was the result of metabolic uptake by the bacteria or due to surface adsorption of the proteins.

A suspension (0·2 ml.) of the test organism (see Smith and Gallop, 1956) was mixed with plasma (0·2 ml.) and gelatin-Locke solution (0·2 ml.), and incubated for ¾ hr. at 37°. The cultures were cooled to 0° and maintained at this temperature for ½ hr. before a solution (0·2 ml.) of one or other of the proteins was added to the test samples and Locke solution (0·2 ml.) to the controls. After standing for a further ¾ hr. at 0° the organisms were collected by centrifugation at 0° and half the organisms washed as described in the previous section. Table IV summarises the results of numerous experiments in which the susceptibility to phagocytosis of the treated organisms (washed and unwashed) was compared with that of control organisms. It was necessary to compare the results obtained with unwashed organisms with those from additional control samples for the following reason. Approximately 1/15th of the original protein mixture remained around the unwashed organisms of the experimental sample and was therefore present during their subsequent phagocytosis. This quantity of material, which would not have affected a normal anti-phagocytic test using recently germinated spores (see Smith and Gallop, 1956), inhibited to some extent the phagocytosis of the organisms described above which had become partially resistant by growing for ¾ hr. in plasma. Furthermore, the degree to which the phagocytosis of these organisms reacted to the residual protein was related to the sample of guinea-

pig plasma in which they were grown. To provide a correct basis for comparison with experimental samples a quantity of protein equal to that trapped around the centrifuged deposit of treated organisms was added to a normal control mixture of blood- and plasma-grown organisms; the phagocytosis of organisms from these samples is included in Table IV as "corrected controls". The results show that *B. anthracis* which has grown in plasma for ¾ hr. can adsorb from proteins A or B a material which confers on them an increased resistance to phagocytosis. Protein B may also contain a little of this material. The adsorption depends to some extent on the preliminary growth of the organisms in plasma since organisms grown in tryptic meat broth for ¾ hr. did not show the phenomenon.

TABLE IV.—*Treatment of Phagocytosis-sensitive Organisms with Proteins A and B at 0°*

Sample.	Conc. (per cent) in contact with organisms at 0°.	Number of experiments from which the average result obtained.	Average per cent of organisms surviving phagocytosis. Washed organisms. (*a*) Control.	(*b*) Expt.	(*b*–*a*).	Unwashed organisms. (*a*) Control.	(*a*) Control (corrected)*	(*b*) Expt.	(*b*–*a*).
Mixture of proteins A and B	0·25	3	11	30	*19*	15	25	41	*16*
Protein A	0·125	11	17	30	*13*	17	24	40	*16*
„ B	0·5	7	32	36	*4*	29	39	44	*5*
	0·25	7	20	28	*8*	21	31	40	*9*

For details of the treatment of organisms see text.
* See text for details of correction.

Protein Fractions from in vitro*-grown Phagocytosis-sensitive Organisms.*

To assess the importance of the protein fractions described above in the resistance to phagocytosis of *B. anthracis in vivo*, phagocytosis-sensitive organisms grown *in vitro* were extracted to see if they contained protein fractions with similar biological activity to those obtained from the resistant organisms. Two strains of *B. anthracis* were grown *in vitro*, the virulent N.P. strain and the avirulent non-capsulated "Sterne" strain. When grown in tryptic meat broth, both strains were readily phagocytosed but they differed in degree of sensitivity. The virulent N.P. strain was 10–20 per cent less susceptible to phagocytosis than the "Sterne" strain when examined in a standard phagocytosis test (Smith and Gallop, 1956); also, relatively higher concentrations of an aggressin were needed to interfere with the phagocytosis of the "Sterne" strain of organisms.

Extracts of the organisms from 16 hr. shaken cultures in tryptic meat broth (see Methods) were separated into five fractions by the method described already for the ammonium carbonate extract of the organisms grown *in vivo*. The extracts were prepared by shaking the organisms with "ballotini" because when grown *in vitro* they do not lyse in ammonium carbonate solution as do those grown *in vivo* (Smith *et al.*, 1953*a*, *b*). Hence a ballotini extract of the latter was prepared and fractionated to permit a strictly comparative study. The yields, analyses and biological activity of the four high M.W. fractions from these extracts

are shown in Table V; corresponding figures for fractions from the ammonium carbonate extract of organisms grown *in vivo* are shown in Table I. It is apparent from Table V that the virulent N.P. organisms formed small amounts of polyglutamic acid when growing *in vitro* in tryptic meat broth but the avirulent uncapsulated " Sterne " strain produced none of this substance.

TABLE V.—*Properties of Fractions from " Ballotini " Extracts of* B. anthracis *Grown* in vivo *and* in vitro

Organism from which extract prepared.	Fraction. Per cent ethanol in barium-acetate ethanol fractionation.	Yield per cent.	Polyglutamic acid per cent.	Anti-phagocytic activity (lowest active conc. per cent).	Anti-complemenary activity (lowest active conc. per cent).
In vivo, N.P.	0 (protein A)	11*	<2	0·07	0·25
	5	6	10–20†	0·5	0·2
	33 (protein B)	14	7	0·16	0·12
	Sol.	9	—	Inactive 0·5	Inactive 2
In vitro, N.P.	0 (protein A)	9*	<2	0·07	0·4
	5	6	7	0·5	0·6
	33 (protein B)	24	3	Inactive 0·5	1·0
	Sol.	4	—	„ 0·5	Inactive 2
In vitro, " Sterne "	0 (protein A)	9*	<2	0·07	1
	5	5	<2	0·5	1·5
	33 (protein B)	19	<2	Inactive 0·5	2
	Sol.	4	—	„ 0·5	Inactive 2

* Proteins A did not go into solution when treated with Zeocarb 215 as easily as did the corresponding fraction from the ammonium carbonate extract of *in vivo*-grown organisms.

† Paper chromatography on hydrolysates showed 15–20 per cent more glutamic acid than in bacterial protein. The paper electrophoretic method indicated approx. 10 per cent polyglutamic acid but some polyglutamic acid appeared to remain with the protein at the origin.

Extracts of the organisms grown *in vitro* contained substances which were active in the anti-phagocytic test but they were present in smaller quantity than in the extracts of organisms grown *in vivo*. Thus, the yield and activity of protein A fractions from organisms grown *in vitro* were similar to those obtained from the organisms grown *in vivo*, but the protein B fractions had negligible activity. In the anti-complementary test, the fractions from N.P. strain grown *in vitro* were less active than those from the *in vivo*-grown organisms while those from the " Sterne " strain were almost inactive.

The different protein fractions were tested for their ability to confer phagocytosis resistance on *B. anthracis* when it was treated with them at 0°. The experiments were made as described in the previous section for proteins A and B from the ammonium carbonate extracts of the organisms grown *in vivo*; the results are summarised in Table VI. Proteins from the virulent organisms grown *in vitro* conferred resistance to phagocytosis but to a smaller extent than those obtained from material grown *in vivo*. On the other hand, treatment with the proteins from the avirulent " Sterne " strain left the test organism phagocytosis-sensitive. Furthermore, in five experiments, organisms of the " Sterne " strain grown in plasma for ¾ hr. were not made more resistant to phagocytosis either by

treatment at 0° with the proteins from N.P. organisms or when these materials were included during the preliminary growth in plasma.

TABLE VI.—*The Treatment of Phagocytosis-sensitive Organisms at* 0° *with Proteins A and B from Extracts of* B. anthracis *Grown* in vivo *and* in vitro

Sample.	Number of experiments from which the average result obtained.	Conc. per cent in contact with organisms at 0°.	Average per cent of organisms surviving phagocytosis. Washed organisms, (a) Control.	(b) Expt.	(b–a).	Unwashed organisms. (a) Control.	(a) Control (corrected).	(b) Expt.	(b–a).
N.P. *in vivo*									
Protein A	3	0·125	23	38	*15*	24	37	50	*13*
„ B	6	0·5	29	36	*7*	31	40	46	*6*
	6	0·25	25	30	*5*	25	25	30	*5*
N.P. *in vitro*									
Protein A	6	0·25	24	30	*6*	17	20	37	*17*
„ B	2	1·0	30	28	*−2*	32	41	43	*2*
"Sterne" *in vitro*									
Protein A	4	0·5	26	26	*0*	31	33	30	*−3*
	10	0·25	28	25	*−3*	26	31	33	*2*
„ B	2	1·0	30	26	*−4*	30	44	44	*0*

DISCUSSION

It is apparent from recent work (Smith and Gallop, 1956; Smith, Tempest, Stanley, Harris-Smith and Gallop, 1956; this paper) that the overall aggressive action of *B. anthracis* growing *in vivo* is not due to a single substance but to an armoury of different products, some produced extracellularly and some forming integral parts of the organism. It seemed improbable that each one would satisfy all tests for aggressive activity that we have used, namely those for anti-phagocytic, virulence-enhancing, anti-complementary and anti-anthracidal activity, and this has proved to be so.

The two most important aggressins which seem to assist *B. anthracis* to establish itself in the tissues of a host are the extracellular toxin (Smith *et al.*, 1955), which later in the disease acts specifically in killing the host, and capsular polyglutamic acid. The toxin is clearly an aggressin because at least one purified component of it has anti-phagocytic, virulence-enhancing, and anti-anthracidal properties (Smith *et al.*, 1956). The following fact emphasises the fundamental importance of the toxin as an aggressin. It and its components are antigens which actively protect animals against the disease, and it is in the very early stages of the latter that the respective antibodies abort the infection (Cromartie, Bloom and Watson, 1947; Cromartie, Watson, Bloom and Heckly, 1947). The production of oedema by the toxin may be responsible for part of its aggressive action (*cf.* Dubos, 1954). Isolated polyglutamic acid which has no toxic or immunising activity is anti-phagocytic and is present in the capsule surrounding *B. anthracis*. Since *in vivo* this material is concentrated at the place where it can be most effective, *i.e.*, between the organism and the phagocyte, its anti-

phagocytic action (Zwartouw and Smith, 1956) probably plays a large part in preventing the ingestion of the capsulated organism. Polyglutamic acid is also found free in the plasma of the host and here it may help to combat any bacteriolytic powers which might be possessed by the latter.

The work described here shows that a further non-toxic factor may contribute to the anti-phagocytic action of virulent *B. anthracis*. It was present in an impure lipoprotein fraction which had a high anti-complementary activity. There is some evidence that this substance, which is of high molecular weight and apparently not connected with the anti-complementary activity, could act at the surface of the organism; it renders phagocytosis-sensitive organisms more resistant to ingestion when they are treated with it at 0°. It was not present in an avirulent uncapsulated strain of *B. anthracis*. Recognition of the possible existence of such a surface material was difficult for two reasons. First, extracts of organisms used in this work were a mixture of somatic and capsular material; it was thus impossible to say whether or not any active fraction was a surface component. Second, protein from virulent capsulated organisms had anti-phagocytic activity yet the bulk of it must have been within the bacillus where it is doubtful that it played any aggressive rôle. Thus the somatic protein from uncapsulated phagocytosis-sensitive organisms was similarly active in the simple anti-phagocytosis test, although it was unable to induce resistance in test organisms after pre-treatment.

The possible addition of a protein fraction to polyglutamic acid as a further anti-phagocytic substance operating in the capsule of *B. anthracis* recalls the relationship between the M protein and hyaluronic acid of capsulated virulent streptococci. Not only is the capsular hyaluronic acid responsible for virulent and anti-phagocytic properties but the M protein, which can be isolated from whole extracts of the organisms, plays its part and some of it is present on the surface of the capsulated organisms (Rothbard, 1948; Morris and Seastone, 1955).

The existence of a possible third component in the aggressive mechanism of *B. anthracis* can only be proved by an examination of the constitution of the capsule of *B. anthracis* if this can be removed preferentially from the somatic material. It is however relevant to point out here that Tomcsik (1956) has suggested that the ability of capsulated *B. anthracis* organisms to produce polyglutamic acid antisera may possibly be due to a connection with protein in the capsule. It should also be remembered that Smith and Gallop (1956) showed that the body fluids of infected guinea-pigs contained a protein aggressin resembling the bacterial protein, in addition to polyglutamic acid from the capsule of the organism.

SUMMARY

A mixture of the capsular and somatic material of *B. anthracis* grown *in vivo* has been extracted and substances with aggressive action isolated.

In addition to capsular polyglutamic acid, which has aggressive activity, some evidence has been obtained that a second aggressin, present in protein fractions, may have an anti-phagocytic action at the surface of the capsulated organism.

The overall aggressive activity of *B. anthracis* is discussed It is due mainly to the action of the extracellular toxin (see previous papers in this series) which later in the disease kills the host, and to capsular polyglutamic acid. The protein

aggressin must be shown to occur in the capsule of *B. anthracis* before its importance in virulence can be assessed.

REFERENCES

Consden, R., Gordon, A. H. and Martin, A. J. P.—(1944) *Biochem. J.*, **38**, 224.

Cromartie, W. J., Bloom, W. L. and Watson, D. W.—(1947) *J. infect. Dis.*, **80**, 1.

Idem, Watson, D. W., Bloom, W. L. and Heckly, R. J.—(1947) *Ibid.*, **80**, 14.

Dubos, R. J.—(1954) ' Biochemical Determinants of Microbial Diseases.' Cambridge, Mass. (Harvard University Press), p. 35.

Flynn, F. V. and de Mayo, P.—(1951) *Lancet*, ii, 235.

Frame, E. G., Russel, J. A. and Wilhelmi, A. E.—(1943) *J. biol. Chem.*, **149**, 255.

Ivanovics, G.—(1940) *Z. ImmunForsch.*, **97**, 402.

Idem and Bruckner, V.—(1937) *Ibid.*, **90**, 304.

Keppie, J., Smith, H. and Harris-Smith, Patricia W.—(1953) *Brit. J. exp. Path.*, **34**, 486.

Markham, R. and Smith, K. M.—(1949) *Parasitology*, **39**, 330.

Morris, M. and Seastone, C. V.—(1955) *J. Bact.*, **69**, 195.

Partridge, S. M.—(1948) *Biochem. J.*, **42**, 238.—(1949) *Nature, Lond.*, **164**, 443.

Rothbard, S.—(1948) *J. exp. Med.*, **88**, 325.

Smith, H. and Gallop, R. C.—(1956) *Brit. J. exp. Path.*, **37**, 144.

Idem, Keppie, J. and Stanley, J. L.—(1953*a*) *Ibid.*, **34**, 471.—(1953*b*) *Ibid.*, **34**, 476.—(1955) *Ibid.*, **36**, 460.

Idem, Tempest, D. W., Stanley, J. L., Harris Smith, Patricia W. and Gallop, R. C.—(1956) *Ibid.*, **37**, 263.

Idem and Zwartouw, H. T.—(1956) *Biochem. J.*, **63**, 447.

Strange, R. E. and Harkness, N.—(1953) *Nature, Lond.*, **171**, 77.

Tomcsik, J.—(1956) *Symp. Soc. gen. Microbiol.* " Bacterial Anatomy." Cambridge (University Press), p. 46.

Zwartouw, H. T. and Smith, H.—(1956) *Biochem. J.*, **63**, 437.

INDUCTION OF VIRULENCE IN AN AVIRULENT STRAIN OF *MYCOBACTERIUM TUBERCULOSIS* BY CERTAIN NON-IONIC SURFACE-ACTIVE AGENTS

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We have previously reported (Hart and Rees, 1954) that when the avirulent strain of *Mycobacterium tuberculosis* H37Ra is subcultivated repeatedly in the presence of certain non-ionic surface-active agents, its typical amorphous growth pattern is replaced by microscopic " cords " similar to those shown by virulent tubercle bacilli; when tested in mice the strain was, however, still avirulent. Similar manipulations of another avirulent strain, H4Ra, have since been shown to result in cord formation, but in this case virulence for mice frequently accompanies the morphological change. In most of the work with H4Ra the commercial agent " Triton A20 " has been used, but this product is no longer obtainable, having been superseded by "Triton WR1339." The latter, although stated to have been the solute of which the earlier " A20 " was merely a 25 per cent aqueous solution, appears to be currently a more refined product; *e.g.*, it lacks the properties of selectively inhibiting the growth of avirulent tubercle bacilli *in vitro* (Rees and Hart, 1952) and of producing convulsions after intravenous administration in large doses to mice. Since cord-forming and virulent strains were only rarely produced from H4Ra by means of Triton WR 1339, even though several batches were tested, we have delayed communicating our findings in detail until reproducibility of the change both in morphology and in virulence could be secured in some other agent besides the extinct Triton A20. This has now been achieved, using the chemically similar, and considerably simpler, surface-active product " D4 ", referred to in previous publications (Cornforth, Hart, Rees and Stock, 1951; Cornforth, Hart, Nicholls, Rees and Stock, 1955; Rees and Hart 1952; Hart and Rees, 1954).

MATERIALS AND METHODS

Surface-active agents

Triton A20, the 25 per cent solution of a polymeric *p-iso*octyl polyoxyethylene phenol, was made by Rohm and Haas Co., Philadelphia. D4 was the dicyclic member (ether of 2, 2′-dihydroxy-5, 5′-di-*tert*-octyldiphenylmethane, with an average of 20 ethylene oxide units per phenolic group) in a series of chemically similar polyoxyethylene ethers synthesised by our colleague Dr. J. W. Cornforth and his co-workers on account of the antituberculous properties of some of them (not D4) in experimental animals (Cornforth *et al.*, 1951; 1955). The agents, in concentrated solution (all concentrations of Triton A20 refer to the effective solute), were sterilised by autoclaving.

Cultures

Myco. tuberculosis, avirulent human strain H4Ra, was obtained from Dr. W. Steenken, Jr., Trudeau Laboratory, New York; it had been isolated by him in 1938 from a culture of a virulent strain obtained from human sputum. The avirulent human strain H37Ra, as well as the virulent H37Rv, were also obtained from Dr. Steenken (see Hart and Rees, 1954).

Media and tests

The egg-yolk agar medium used for maintenance of the strain and for most of the tests, and " the liquid medium " (containing 0·5 per cent albumin (bovine plasma fraction V) and the required amount of Triton A20 or D4) used for submerged growth, have been described previously (Hart and Rees, 1954). For the " main streams " (see later), cultures 3 or more weeks old on egg-yolk agar were ground in Griffith tubes to give aqueous suspensions equal in opacity (by eye) to a standard containing 0·2 mg. (wet-weight)/ml., and 0·2 ml. of these suspensions, or of a 1/10 dilution thereof, was inoculated into 25 mm. diameter test tubes containing 5 ml. of the liquid medium with surface-active agent. After incubation at 37° for 2–3 weeks, 0·2–0·4 ml. was inoculated into the next tube of the liquid medium, and so on. For the " secondary streams," the inoculum was 1·0 ml. culture from one of these serial tubes into 50 ml. liquid medium in 250 ml. conical flasks, and the latter were incubated for 9 days, being shaken manually by circular motion twice daily to promote dispersion. Secondary egg-yolk agar cultures were derived by inoculation from the bacterial deposit of liquid medium cultures, or from mouse lung homogenates, and incubation for 2–3 weeks.

Examination of cultures

The presence of granular or dispersed submerged growth in the liquid medium was observed by the naked eye, or with a hand lens, after gentle shaking. Microscopic growth pattern was assessed by the appearance of preparations stained by the Ziehl-Neelsen method. Colonial morphology was assessed by sowing samples of suitably diluted suspensions of cultures, grown either in the liquid medium or on egg-yolk agar, on the surface of plates of oleic-albumin agar medium, which were then incubated in plastic bags for 3–4 weeks and observed by incident light at a magnification of × 25.

Inoculation of mice (virulence tests)

Suspensions were prepared from 9-day cultures in the flasks or from 2-week egg-yolk agar cultures, as described previously (Hart and Rees, 1954), the dose injected intravenously being, respectively, 0·2 mg. or 0·1 mg. wet-weight bacilli. Female (18–20 g.) mice of the albino P strain were used. Virulence was assessed by tuberculosis mortality (confirmed by autopsy) and median survival time (defined in a footnote of Table I) during 35 days following infection, at which time tests were terminated ; assessment by extent of pulmonary involvement was found to be unsatisfactory. Under these conditions, the median survival time for the standard virulent strain H37Rv was, on the average, 18 days, with all dead by 35 days.

Dehydrogenase activity (methylene blue decolouration tests)

The standard Thunberg method was used (Umbreit, Burris and Stauffer, 1945) with glucose as substrate and with 10 mg. (wet-weight) washed bacilli for each estimation. The source of the bacilli was either the liquid cultures (in Triton A20) used for study of colonial morphology or virulence, or cultures specially grown in " Tween "-albumin medium from an egg-yolk agar culture, or, in a few instances, from a culture on Proskauer and Beck's defined medium.

RESULTS

Production of Virulent Strains from the Avirulent H4Ra, using Triton A20

Samples of suspensions (preferably diluted 1/10) prepared from well-grown egg-yolk agar cultures of H4Ra were inoculated into tubes of the liquid medium containing Triton A20 0·002 per cent. After incubation, samples from these tubes were seeded into tubes of the medium containing 0·05 per cent agent, growth being now obtained in this concentration whereas initially it usually failed owing to the agent's characteristic inhibitory effect on avirulent tubercle bacilli (sees Rees and Hart, 1952). The process of transfer and culture was repeated several times in 0·05 per cent agent, accompanied by microscopic examination at each stage. At various points in this " main stream ", "secondary streams " were made by subcultivating from 0·05 per cent serial tube cultures into flasks of

50 ml. of the liquid medium containing 0·0005, 0·001 or 0·05 per cent agent (concentrations of 0·0005 and 0·001 per cent were obtained by adding 0·5 and 1·0 ml. culture, respectively, to liquid medium devoid of agent, thus carrying over in the inoculum the required amount of the latter); these secondary liquid cultures were examined microscopically and plated to show colonial morphology, and they, as well as egg-yolk agar cultures derived from them, were tested for virulence.

The base-line findings and progress of a typical experiment are shown in Table I (information on cultures in 0·0005 per cent agent is omitted). It will be seen

TABLE I.—*Changes in Microscopic Growth Pattern, Colonial Morphology and Virulence of Strain H4Ra after Serial Subculture in "Triton A20"*

	Main stream.		Secondary streams.			
	Microscopic growth pattern.	Medium.	Medium.	Microscopic growth pattern.	Colonial* morphology.	Virulence: Tb. mortality.§
Base-line	—	Egg-yolk agar	Egg-yolk agar	—	—	0/12 (—)
			LM† (0·001% agent)	Amorphous	—	—
	Amorphous	LM (0·002% agent)	LM (0·002% agent)	,,	No S‡	0/12 (—)
	,,	LM (0·05% agent)	LM (0·001% agent)	,, + cords	50–80% S	(See p. 376)
	,,	Ditto				
	,,	Ditto	LM (0·001% agent)	Cords	—	4/12 (—)
	,, + sparse cords	Ditto				
	Amorphous + sparse cords	Ditto	LM (0·05% agent)	Amorphous	>99% S	5/12 (—)
			LM (0·001% agent)	Cords	>99% S	8/12 (24)
	Amorphous	Ditto	LM (0·05% agent)	Amorphous	100% S	8/12 (23)
			Egg-yolk agar	—	—	7/12 (29)
			LM (0·001% agent)	Cords	100% S	11/12 (20)
			Egg-yolk agar	—	—	9/12 (28)

* On oleic-albumin agar plates.
† LM . . The liquid medium (see p. 373).
‡ S . . Spreading (rough) colonies: remainder are circumscribed (smooth) colonies.
§ Mortality expressed as mice dying of tuberculosis/total mice; median survival time (*i.e.*, number of days after infection by which half the mice had died of tuberculosis) in brackets. Experiments terminated 35 days after infection.

that although there was little or no development of cords in the serial cultures in 0·05 per cent agent (though in some experiments cord formation was prominent even at this concentration), secondary cultures therefrom in 0·001 per cent (and still more so in 0·0005 per cent) showed definite cords, suggesting masking at the higher concentration. Virulence, absent in the base-line tests, became evident in the cultures in 0·05 per cent and to a greater degree in those in the lower concentrations ; it was also shown in the secondary egg-yolk agar cultures. The morphology changed from apparently 100 per cent circumscribed (smooth) colonies, in a base-line culture, to a mixture of 50–80 per cent spreading (rough) and 20–50 per cent circumscribed colonies at an early stage of progress, and finally to 100 per cent of spreading type ; and it is important to note that the colonial change, like virulence, applied to plates seeded from cultures in 0·05 per cent agent as well as from those in the lower concentrations (in some other experiments it was tested and shown also on plates inoculated from secondary egg-yolk agar cultures).

Animals infected with induced-virulent strains of H4Ra tended to become ill and thin in about 2 weeks ; thereafter some might recover but in those that died the lesions were usually confined to the lungs and frequently " semi-chronic " in character. (In series in which significant virulence, as judged by mortality, failed to develop, many of the mice still became ill and thin but all had recovered by the termination of the experiment ; by contrast, mice infected with base-line cultures invariably remained healthy and gained weight.)

The lethal effect of cultures in the lower concentrations (0·001 or 0·0005 per cent) of agent seemed to vary with the granularity of growth in the particular case ; support for this association was obtained from an experiment secondary to that in Table I. A serial culture in 0·05 per cent agent, which showed well dispersed growth and gave a mortality of 5/12 mice, was subcultivated in 0·0005 per cent agent. The mortality in mice infected with the latter, granular, culture was raised to 11/12 mice (median survival 14 days). The granular culture was further subcultivated both into 0·0005 per cent and into 0·05 per cent, giving granular and dispersed growth respectively. The resulting mortalities in mice inoculated with the former was 11/12 (median 10 days), similar to previously, while that of the latter was 6/12 (median 33 days), having fallen to the level of the original culture in 0·05 per cent agent. At the same time, the growth pattern, which had been largely amorphous in the original 0·05 per cent culture but had shown well-marked cord formation in the 0·0005 per cent subcultures, reverted to the previous largely amorphous character after return to 0·05 per cent. These findings also confirm that the apparent lack of cords in some " streams " after serial subculture in 0·05 per cent agent is probably due to masking and not to true absence in this concentration.

For comparative tests of virulence, the most useful cultures are those on egg-yolk agar derived from cultures in 0·05 or 0·001 per cent agent, more simply from the former. The ground suspensions from such solid medium cultures are comparable, in colonial morphology as well as in virulence, directly with the base-line egg-yolk agar cultures, without the complicating variable of granularity.

The rate and degree of cord formation in H4Ra after the treatment with surface-active agent varied from one " stream " to another, and it should be emphasised that not all produced virulent strains. Moreover, in the experiment in Table I, the development of virulence appeared to be gradual as serial cultures in 0·05 per cent agent proceeded, reaching a peak ; in some other experiments the

emergence was rapid and maximal at an early stage. It was difficult to foretell the degree of virulence in a treated strain from the degree of cord formation either in 0·05 per cent or in 0·001 or 0·0005 per cent Triton A20, beyond the fact that if cords were absent altogether in the low concentrations so also was virulence; certainly definite cord formation might be observed without virulence. On the other hand, there was a suggestion that the degree to which the plated colonies approached, in quantity and in character, those obtained from the standard virulent strain H37Rv permitted a closer estimate of the degree of virulence.

Stability of the virulence

That the induced virulence of H4Ra persists after transfer to medium from which Triton A20 is absent is already evident from the fact (Table I) that egg-yolk agar cultures derived from virulent cultures in the agent were able to kill mice; so also were subcultures in Tween-albumin medium of such secondary egg-yolk agar cultures. In another experiment, the bacterial deposit from a "secondary stream" culture in Triton A20 0·001 per cent, which had given a mortality of 12/12 mice (median survival 18 days), was sown on egg-yolk agar, and the resulting culture, which itself also killed an entire group of mice (median 19 days), was subcultivated a further 5 times on the solid medium. Suspensions prepared from the final culture were tested in mice, giving again 12/12 deaths (median 20 days); moreover, this suspension showed 100 per cent spreading colonies when plated.

From these results it is not surprising that cultures recovered from the lungs of mice infected with virulent H4Ra were also virulent; indeed, passage resulted usually in somewhat raised virulence, though even now extrapulmonary lesions, common in early deaths after infection with the virulent strain H37Rv, were few or absent and the lesions tended to be "semi-chronic" in nature. A single passage in mice of unmodified H4Ra failed to affect its avirulence or to change its colonial morphology.

Stability of differentiation between spreading (rough) and circumscribed (smooth) colonies

From the oleic-albumin agar plates (Table I) showing a mixture of the spreading (S) and the circumscribed (C) types of colony at an early stage of progress, representative single colonies were picked off and transferred to tubes of the liquid medium containing Triton A20 0·001 and 0·002 per cent. The morphology of the resulting liquid cultures was in strong contrast. S showed cords microscopically, and entirely the spreading type of colony when plated, whereas C showed

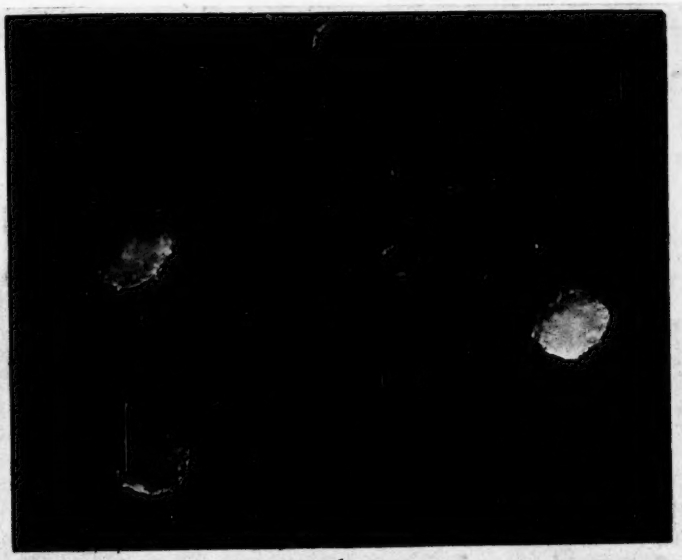
EXPLANATION OF PLATE

Contrasting colonial appearance, on oleic-albumin agar plates, of substrains of H4Ra obtained during an early stage of treatment with surface-active agent (× 40) (see text, p. 376).

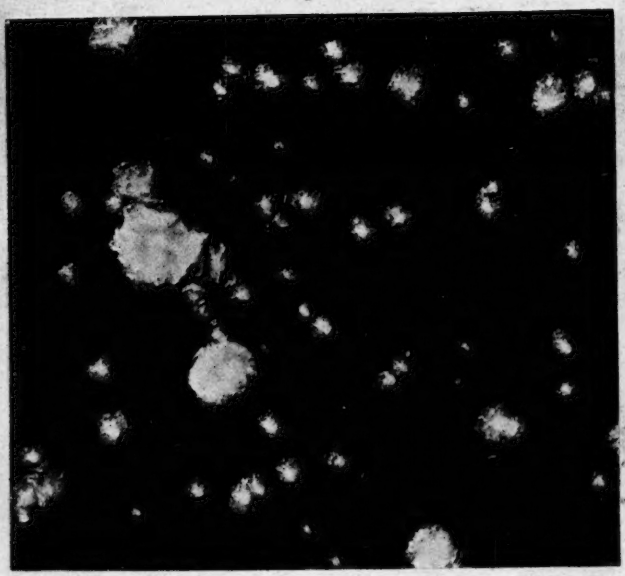
1. Entirely circumscribed (C) colonies; the substrain had been obtained, *via* liquid medium, from a single circumscribed colony on a plate carrying a mixture of spreading and circumscribed types, which had originated from the first serial subculture of H4Ra in Triton A20 0·05 per cent.

2. Entirely spreading (S) colonies; the substrain had been similarly obtained from a single spreading colony on a plate carrying a mixture of the two types.

When tested in mice, the original unmodified strain was avirulent, the C substrain also avirulent, and the S substrain virulent.



1



2

D'Arcy Hart and Rees.

an amorphous growth pattern and entirely the circumscribed type of colony (Plate). So also were virulence tests of the substrains in contrast, S killing 12/12 mice (median survival 23 days) and C killing none. Hence, after isolation and subculture, the spreading and circumscribed colonies of the mixture had maintained their distinctness in appearance and in characteristic effect in mice. Biochemical distinctness of these substrains was also retained, for the methylene blue decolouration time (see later) of test cultures from the S colonies was 90 min., resembling that for H37Rv, whereas the decolouration time of cultures from the C colonies was 11 min., resembling that of the base-line H4Ra (12 min.).

Part played by albumin

The liquid medium used for the development of cords and virulence in H4Ra contains albumin 0·5 per cent, and it was possible that this ingredient favoured these changes. It is not known whether the changes would take place if the medium contained surface-active agent but no albumin. On the other hand, tests have been made in the presence of albumin without agent. Well-grown egg-yolk agar or Proskauer and Beck cultures of H4Ra were subcultivated repeatedly in the liquid medium containing albumin but devoid of agent. At the first culture the colonial appearances on oleic-albumin agar plates were substantially unaltered, almost all the colonies being circumscribed and typical of unmodified H4Ra, though there was an occasional one intermediate in appearance between the circumscribed and spreading types. After 6–10 serial subcultures in the albumin medium (by which time cord formation had already been observed in parallel tubes containing Triton A20 as well as albumin), there was evidence of slight cord formation microscopically ; moreover, plates from such liquid cultures or from egg-yolk agar cultures derived therefrom were unusual in appearance, showing a mixture of circumscribed and intermediate-type colonies, with an occasional spreading colony typical of the induced-virulent strains of H4Ra. These intermediate morphological changes were, however, not accompanied by development of virulence, since neither suspensions from ground centrifuged deposits nor those from secondary egg-yolk agar cultures killed mice, nor did cultures recovered from the lungs of these infected animals and tested in fresh groups. Moreover, intermediate-type colonies reverted to typical circumscribed colonies on subculture and re-plating. Hence it was concluded that while the albumin in the liquid medium might facilitate the changes in morphology and virulence induced in the presence of Triton A20, *e.g.*, by assisting in selection of variants, it was not the sole factor.

Virulence for guinea-pigs

Guinea-pigs were injected subcutaneously with 0·1 mg. wet-weight bacilli from a liquid culture of H4Ra showing cords and spreading colonies, and at the same time mice were infected intravenously with 0·2 mg. On termination of the experiments at 6 or 12 weeks, the guinea-pigs showed some macroscopic visceral involvement, but progression was limited. No systemic spread was evident on macroscopic or microscopic examination of controls infected with " base-line " cultures of H4Ra showing amorphous growth pattern and circumscribed colonies ; abnormal appearances were confined to local lesions at the site of inoculation. The mice had a mortality of 10/12 during 35 days, as compared with *nil* in their

controls. Cultures recovered from a guinea-pig infected with an induced-virulent strain of H4Ra had retained their virulence for mice, killing 12/12. It was concluded from these experiments that treated H4Ra was virulent for guinea-pigs, but less so than for mice.

Production of Virulent Strains from H4Ra, using D4

In an experiment modelled on that in Table I, but using the agent D4 instead of Triton A20, 0·2 ml. of a 1/10 dilution of a standard suspension prepared from a 3 week-old egg-yolk agar culture of H4Ra was added to tubes of the liquid medium containing D4 0·002 per cent and 0·05 per cent. After incubation for 2 weeks, growth was evident in the former but characteristically failed in the latter (Rees and Hart, 1952). The culture in D4 0·002 per cent was seeded in 0·4 ml. amount into a tube of the medium containing D4 0·05 per cent, growth being now obtained, and two further serial subcultures were made at 2-weekly intervals in this concentration of agent. Secondary liquid and egg-yolk agar cultures were made in the usual manner. The results of microscopic and colonial examinations, as well as of virulence tests, are shown in Table II. A striking contrast is evident

TABLE II.—*Changes in Microscopic Growth Pattern, Colonial Morphology and Virulence of Strain H4Ra after Serial Subculture in "D4"*

	Main stream.		Secondary streams.			
	Microscopic growth pattern.	Medium.	Medium.	Microscopic growth pattern.	Colonial* morphology.	Virulence: Tb. mortality.§
Base-line	—	Egg-yolk agar	→ Egg-yolk agar	—	No S‡	0/12 (—)
	Amorphous + occasional cord	LM† (0·002% agent)				
	Amorphous + sparse cords	LM (0·05% agent)				
	Amorphous + cords	Ditto	→ LM (0·001% agent)	Cords	>99% S	10/12 (22)
	Amorphous + sparse cords	Ditto	→ Egg-yolk agar	—	>99% S	7/12 (29)

Key as Table I.

between the almost universally spreading type of colony and the virulence of the secondary egg-yolk agar culture derived after serial subculture in D4 0·05 per cent, on the one hand, and the typically circumscribed colonies and lack of virulence of the base-line culture on the same solid medium, on the other. Cord formation was masked in 0·05 per cent agent, but evident in the low concentration (0·001 per cent) of the secondary liquid culture. These results support and extend those obtained with Triton A20.

Dehydrogenase Activity

The results of the tests of dehydrogenase activity of washed suspensions of tubercle bacilli, as measured by methylene blue decolouration times, are given in Table III. The unmodified, amorphous-growing, avirulent strains H4Ra and H37Ra

TABLE III.—*Methylene Blue Decolouration Times for Certain Strains of* Myco. tuberculosis *before and after Treatment by Serial Subculture in Surface-active Agent* (H4Ra and H37Ra), or before and after Attenuation (H37Rv).*

	Strains.	Microscopic cord formation.	Virulence: Tb. mortality in mice.	Methylene blue decolouration times in individual tests (min.).†
H4Ra	Unmodified strain	0	0	12, 13, 15, **20**, 20, 25
	Treated strains	++ or +	++, + or 0	**33**, 33, 40, 63, >60, >60, >60, >60, >60, >60, 90
	C Substrain ‡	0	0	11
	S „	++	++	90
H37Ra	Unmodified strain	0	0	16, **19**, 20, 22
	Treated strains	++	0	5, **17**, 22, 24
H37Rv	Unmodified strain	+++	+++	50, 50, 58, >**60**, >60, >60, >60, 90
	Attenuated "DH"	+++	0	28
	„ "VAL"	+	0	**16**, 20
	INH-resistant	+++	0	>**80**, >90

* Triton A20 or D4.

† Tests are arranged in order of increasing magnitude, median values being in bold type.

‡ C and S substrains were derived, respectively, from single Circumscribed (smooth) and Spreading (rough) colonies, which had been obtained in mixture by plating a partially treated strain of H4Ra (see p. 377).

decolourised rapidly (median times 20 and 19 min. respectively), in contrast to the unmodified virulent strain H37Rv (median time greater than 60 min.). After serial subculture in surface-active agent, the decolouration times of cord-forming, virulent strains derived from H4Ra were lengthened (median value greater than 60 min.), while those of cord-forming but still avirulent strains derived from H37Ra were unchanged (median 17 min.). To make a closer analysis, a culture of H4Ra was plated out at an early stage in the serial subculture in agent. A mixture of circumscribed (C) and spreading (S) colonies was obtained, and single colonies were picked off to give respective C and S substrains; their decolouration times were quite distinct (11 min. for C and 90 min. for S).

For additional interest some strains modified from H37Rv are included. Two of these ("DH" and "VAL"), having attenuated virulence, had been derived by chance cultural procedures. One retained good, and the other now showed poor, cord formation; both decolourised more rapidly (28 min. and 16 min.) than the unmodified parent strain. A highly isoniazid-resistant, catalase-negative strain, having low virulence for guinea-pigs and mice, had been obtained by manipulation *in vitro*; its decolouration time was the same as that of unmodified H37Rv, suggesting a different mechanism from the processes involved in the other virulence changes under consideration here.

To investigate whether the differences in dehydrogenase activity between virulent and avirulent organisms is due to differences in the amount of intracellular enzyme, certain strains were tested *in the presence of* surface-active agent, in a concentration which might be expected to increase the permeability of the cell surface. Triton A20 was added to the substrate in the Thunberg tube to give a final concentration of 0·14 per cent, and the test was carried out otherwise in the usual manner. Control tests without the bacteria showed that the agent did not itself decolourise the dye. Under these conditions the decolouration time of unmodified H37Rv was reduced from 90 min., in the absence of the agent, to 29 min. when it was present during the test ; the decolouration time for unmodified H4Ra was similarly reduced from 20 min. to 8 min. These results indicate that the dehydrogenase content of virulent organisms is not particularly low, but that the state of cell permeability is an important factor in the activity shown in the methylene blue test by various cultures.

DISCUSSION

There are two morphological accompaniments to the development of virulence in H4Ra during treatment with these surface-active agents, namely, (1) change in microscopic growth pattern from an amorphous character to the formation of " cords ", and (2) change in colonial morphology from circumscribed (smooth) colonies to spreading (rough) colonies. Appearances approaching those of standard virulent strains of *Myco. tuberculosis* thus replace appearances characteristic of avirulent strains. Of the two morphological changes, the second is the more reliable gauge of progress. Cord formation tends to be masked in the liquid medium containing the higher concentration of agent, and needs transfer to lower concentrations for demonstration. Colonial analysis, on the other hand, is independent of the concentration of agent, and it can be applied to cultures on solid as well as in liquid medium. Changes in the relative proportion of colonies of circumscribed, spreading, or intermediate type can be readily assessed.

The development of virulence in H4Ra under the conditions described is irregular ; in spite of changed morphology it failed to occur in 1 out of 4 series in Triton A20 and in 1 out of 3 series in D4. When induced, however, the virulence (as well as the changed colonial morphology) is maintained even when the strain is subcultivated several times on egg-yolk agar devoid of surface-active agent. A single passage in mice may show some further enhancement, although the semi-chronic nature of many of the pulmonary lesions distinguishes from usual virulent strains of *Myco. tuberculosis*. The stability of virulence and changed morphology induced in H4Ra appears to be greater than that of the cord pattern of H37Ra ; as previously reported (Hart and Rees, 1954), the latter gradually reverts to the amorphous pattern of the unmodified strain after a few subcultures in the absence of surface-active agent.

The stability of the induced virulence in H4Ra is also shown by following the differentiation into spreading (and cord-forming) and circumscribed (and non-cord-forming) types of colony which, in an early stage of the process, were evident as a mixed culture on the plates. Isolation and subculture from single colonies showed that these two types reproduced themselves and that the corresponding substrains were, respectively, virulent and avirulent when used to infect mice. This finding differs from the experience in the colonial analysis of certain other mycobacterial

strains. Thus Suter and Dubos (1951) found that different strains of B.C.G. produced characteristic proportions of spreading (cord-forming), non-spreading (non-cord-forming) and intermediate-type colonies on oleic-albumin agar; but although these proportions remained fairly stable during cultivation *in vitro* and animal passage, and although the spreading and non-spreading substrains tended to reproduce themselves, the substrains derived from a given strain could not be distinguished either from each other or from their parent by differences in virulence for experimental animals. Similarly, Linell and Nordén (1954) dissociated a strain of *Mycobacterium balnei* into rough and smooth colonies, the separation persisting on subculture and animal passage; but the virulence for mice of cultures derived from the two types was equal. Variation in B.C.G. and *Myco. balnei* seems to be of a different order from that in H4Ra.

The mortality in mice infected with virulent H4Ra grown in liquid culture containing 0·0005 or 0·001 per cent Triton A20 or D4 is usually greater and earlier than that when a liquid culture containing 0·05 per cent agent, or a suspension from a culture on solid medium, is used. This difference is probably due to the greater granularity of the cultures in low concentrations of agent, and the consequent frequency of embolic foci of tuberculous pneumonia, such as have been described in mice after intravenous injection of clumpy suspensions of virulent bacilli (Mayer, Jackson, Whiteside and Alverson, 1954). It may be noted that granular cultures of *un*modified H4Ra in low concentration of surface-active agent give rise to no mortality in mice, thus excluding embolic foci *per se* as the cause of the virulence of treated strains.

Instances of enhancement of virulence of avirulent tubercle bacilli have been observed under circumstances different from the present. Frimodt-Möller (1939) described two eugonic much attenuated variants, recently dissociated from dysgonic virulent bovine strains, which recovered their original high virulence after a single animal passage, although maintaining their eugonic growth characteristics. On the other hand, unmodified H4Ra retains its avirulence as well as its typical colonial morphology after a single mouse passage, and appears to require *in vitro* manipulation to yield a virulent strain (with changed morphology).

While apparently spontaneous cord formation, multiplication *in vivo*, and even gross lesions have been noted occasionally by others for the avirulent strain H37Ra (see discussion by Hart and Rees, 1954), there are no such reports of spontaneous variation for H4Ra, though, admittedly, the latter has been less studied. On the other hand, using surface-active agents, it is in H4Ra and not in H37Ra that virulence has now been artificially induced.

The effect on H4Ra of the procedure used, that is of serial subculture in the surface-active agents, offers, indeed, an interesting contrast with our experience previously reported for H37Ra (Hart and Rees, 1954). It is true that the present method of treatment by repeated subculture in 0·05 per cent agent differs from the subculture in 0·001 per cent previously used for H37Ra, but it has been applied also (in unreported experiments) to that strain, inducing cord formation (accompanied by spreading colonies) more consistently than by the earlier method; moreover the cords are usually more striking in appearance than with H4Ra. Yet the treated, cord-forming, H37Ra has still failed to show significant virulence even by this method, suggesting that mere cord formation does not determine virulence, at least in some strains of mycobacteria. It is, of course, possible that further modifications of treatment might induce virulence in H37Ra. Further-

more, nothing in the present observations controverts the view that, among mammalian tubercle bacilli, " while all cord-formers are not necessarily virulent, all virulent strains are cord-formers " (Yegian and Kurung, 1952).

In our previous paper a hypothesis was mentioned to account for the mechanism of cord formation by H37Ra after treatment with Triton A20 or D4, namely that these particular agents, by inhibiting (as they do) the growth of amorphous avirulent organisms and by permitting the growth of cord-forming mutants, favour selection of the latter during serial subculture. This hypothesis was then considered unlikely for several reasons, particularly that cord formation could be induced in the presence of other agents which had no growth-inhibitory action either on avirulent or on virulent organisms. However, the fact that, in H4Ra, growth-permitting agents only rarely promote effectively cord formation and the development of virulence, whereas the growth-inhibitory Triton A20 and D4 frequently do so, makes it necessary to reconsider this hypothesis, though possibly only to account for an accessory surface effect of the agents. In this connection it is relevant that the polycyclic mixture Triton A20 contains significant amounts of the dicyclic compound D4. It should be added that no spreading colony has been observed on plates from unmodified H4Ra.

The ability to alter the growth pattern, with or without alteration of the virulence, of certain strains of *Myco. tuberculosis* deliberately *in vitro* offers a means of studying correlative properties better than by merely comparing naturally-differing strains. A number of investigators have observed that the rate of decolouration of methylene blue, using the Thunberg method, by washed suspensions of mycobacteria is apparently related to their virulence (Aksianzew, 1933; Roulet, Wydler and Zeller, 1946; Bloch, 1950*a*; Desbordes and Fournier, 1950). The present experiments provide a special instance of this relationship. Unmodified amorphous-growing H4Ra decolourises the dye rapidly, as is characteristic of avirulent strains of tubercle bacilli; whereas, after cord formation, accompanied usually by virulence, has been induced by treatment with Triton A20 or D4, the reduction time is prolonged and of the same order as that for standard virulent strains. By contrast, strains developed from H37Ra, by similar treatment, to show well-marked cord formation, but no virulence, retain the property of rapid decolouration shown by the unmodified parent strain. Dehydrogenase activity of these tubercle bacilli appears therefore to be more closely related than cord formation to the development of virulence. This inference is supported by the observation that two attenuated strains derived from the virulent H37Rv by cultural procedures gave decolouration times shorter than those of the parent strain; yet one showed poor and the other well-marked cord formation.

The present observations afford opportunity also to study another property of tubercle bacilli which is believed to be associated with their virulence, namely the possession of a chemical constituent " cord factor " (Bloch, 1950*b*; Bloch and Noll, 1955; Noll and Bloch, 1955; Asselineau and Lederer, 1955). Clearly it would be of interest to compare the content of cord factor, both in H37Ra and in H4Ra, before and after treatment with surface-active agent has induced cord formation and, in the case of H4Ra, virulence. Dr. Hubert Bloch and his colleagues have undertaken such a study, using some of our strains.

Bloch (1950*a*) has shown that, although inactive, the dehydrogenase is not absent in suspensions of virulent tubercle bacilli, since they become capable of

decolourising methylene blue after disintegration by grinding, extraction with petroleum ether, or after culture in medium containing a high concentration of Tween 80. These findings are supported by our experiment showing that suspensions of virulent bacilli did decolourise the dye rapidly if they were exposed to Triton A20 *during* the dehydrogenation test. Bloch suggests that relative impermeability of the lipid cell surface, retarding contact between substrate and enzyme, accounts for the usual lack of demonstrable dehydrogenase activity in suspensions of virulent organisms. According to our present experience with the two avirulent strains—in which treatment with surface-active agent induces the formation of cords in both but virulence in only one—it is in the latter that a concomitant diminution or suppression of dehydrogenase activity is observed. In other words, when cord formation is present and apparently held constant, dehydrogenase activity varies inversely with virulence. This suggests the possibility that if permeability of the bacterial cell surface is the link between these two variables, it may involve chemical constituents additional to those, such as cord factor, associated with cord formation.

SUMMARY

When the avirulent strain of *Mycobacterium tuberculosis* H4Ra was repeatedly subcultivated in a specified manner in a liquid medium containing Triton A20 or the chemically similar, but simpler, non-ionic surface-active polyoxyethylene ether D4 synthesised in this Institute, the morphology changed from the amorphous growth pattern and the circumscribed type of colony characteristic of avirulent tubercle bacilli to the formation of microscopic " cords " and spreading colonies similar to those shown by virulent strains such as H37Rv. Cord-forming strains developed by this treatment from H4Ra frequently produced a mortality when tested in mice ; in this respect they differed from cord-forming H37Ra produced by similar treatment, whose virulence was as low as that of the unmodified parent strain.

The induced virulence of H4Ra persisted after transfer to, and subculture in, medium devoid of surface-active agent ; cultures recovered from the lungs of infected animals showing progressive disease were also virulent. Substrains from single spreading and circumscribed colonies, which were present in mixture on plates prepared from a culture at an early stage of the progress of treatment of H4Ra with surface-active agent, were sharply differentiated by virulence as well as by morphological and biochemical characteristics.

Washed suspensions of unmodified, amorphous-growing, H4Ra decolourised methylene blue rapidly in the usual manner of avirulent tubercle bacilli ; cord-forming, virulent strains produced therefrom by treatment with surface-active agent showed the low dehydrogenase activity (delayed decolouration of the dye) of standard virulent strains. Suspensions of cord-forming (but still avirulent) strains developed similarly from H37Ra retained the property of rapid decolouration possessed by the parent strain. Dehydrogenase is not absent in suspensions of virulent organisms, but contact between enzyme and substrate is probably retarded by a relatively low permeability of the bacterial cell surface.

The mechanism of the induction of virulence in H4Ra, and the relationships of cord formation, dehydrogenase activity, and virulence in tubercle bacilli, are discussed. Mere cord formation does not appear to determine virulence, although all virulent strains appear to be cord-formers. Dehydrogenase activity in the

strains studied appears to be more closely related to virulence than is cord formation. If cell permeability is the link between such activity and virulence, the possibility is suggested that other chemical cell constituents besides those associated with cord formation are involved in the permeability changes.

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REFERENCES

AKSIANZEW, M. I.—(1933) *Z. Tuberk.*, **68**, 249.
ASSELINEAU, J. AND LEDERER, E.—(1955) *Biochim. biophys. Acta*, **17**, 161.
BLOCH, H.—(1950*a*) *Amer. Rev. Tuberc.*, **61**, 270.—(1950*b*) *J. exp. Med.*, **91**, 197.
Idem AND NOLL, H.—(1955) *Brit. J. exp. Path.*, **36**, 8.
CORNFORTH, J. W., HART, P. D'ARCY, NICHOLLS, G. A., REES, R. J. W. AND STOCK, J. A.—(1955) *Brit. J. Pharmacol.*, **10**, 73.
Idem, HART, P. D'ARCY, REES, R. J. W. AND STOCK, J. A.—(1951) *Nature, Lond.*, **168**, 150.
DESBORDES, J. AND FOURNIER, E.—(1950) *Ann. Inst. Pasteur*, **79**, 210.
FRIMODT-MÖLLER, J.—(1939) ' Dissociation of Tubercle Bacilli.' London (H. K. Lewis & Co.).
HART, P. D'ARCY AND REES, R. J. W.—(1954) *J. gen. Microbiol.*, **10**, 150.
LINELL, F. AND NORDÉN, A.—(1954) *Acta tuberc. scand.*, Suppl. 33.
MAYER, E., JACKSON, E. R., WHITESIDE, E. S. AND ALVERSON, C.—(1954) *Amer. Rev. Tuberc.*, **69**, 419.
NOLL, H. AND BLOCH, H.—(1955) *J. biol. Chem.*, **214**, 251.
REES, R. J. W. AND HART, P. D'ARCY—(1952) *J. gen. Microbiol.*, **7**, 372.
ROULET, F., WYDLER, H. AND ZELLER, E. A.—(1946) *Helv. chim. Acta*, **29**, 1973.
SUTER, W. E. AND DUBOS, R. J.—(1951) *J. exp. Med.*, **93**, 559.
UMBREIT, W. W., BURRIS, R. H. AND STAUFFER, J. F.—(1945) ' Manometric Techniques,' p. 126. Minneapolis (Burgess Publishing Co.).
YEGIAN, D. AND KURUNG, J.—(1952) *Amer. Rev. Tuberc.*, **65**, 181.

THE BACTERICIDAL ACTION OF TYPHOID ANTIBODY

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THE study of the bactericidal action of serum on bacteria has progressed slowly, partly because of the complex nature of serum, in which there are several antibacterial mechanisms, and partly because of misleading results due to the methods of study employed. The present work was undertaken to consider only one bactericidal system, that of antibody and complement, with the object of measuring the bactericidal action of antibody on *Salmonella typhi*.

When normal antibody is under consideration it is especially difficult to be certain that only a single reaction is being observed because relatively concentrated serum has to be employed. With immune antibody much greater dilution of the serum is possible so that confusion is unlikely to be produced by such serum constituents as properdin (Pillemer, Blum, Lepow, Ross, Todd and Wardlaw, 1954).

In most of the methods which have been used the bacterial suspension has been exposed to antibody and complement for a variable length of time and the extent of the reaction assessed by viable counts. The influence of agglutination on bacterial counts has long been known. Simultaneous mixture of antibody, complement and bacteria (Adler, 1953) may result in competition for complement between the bacterial antigen and soluble antigen, especially when absorbed guinea-pig serum is used as the source of complement. With prolonged reaction times the growth of surviving organisms may obscure the results of an initial bactericidal effect. The results of experiments with stock laboratory cultures may be very different from those given by recently isolated virulent strains; and inocula containing many organisms are not strictly comparable with the small number of organisms found in the blood in typhoid fever (Pulvertaft, 1930; Watson, 1955).

In the experiments to be described the action of immune antibody was considered as an elementary system of three components: antigen, *i.e.*, the bacterial cell, antibody and complement. To avoid competition for complement the cells were first exposed to antibody in order to sensitise them and then complement was added. The extent of bactericidal activity was measured simply by noting the extent to which the inocula were killed. Descendants of survivors were made readily visible by the reduction of tetrazolium salt, 2, 3, 5-triphenyl tetrazolium chloride. In this way a simple titration technique was evolved in which the time saved in performing viable counts could profitably be used to perform more complex experiments.

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MATERIALS AND METHODS

Apparatus

Titrations were performed on standard M.R.C. Perspex haemagglutination trays which contain 10 rows of 8 cups each 1·5 cm. in diameter. Sterile trays were kept covered with a sterile sheet of glass cut to size. To prevent contamination when adding the reagents a mobile Perspex cover was devised consisting of a sheet of Perspex 30 × 40 cm. and 1 cm. thick, which was mounted on small ball-bearing castors so that it just cleared the top of the tray when the glass cover was removed. In the centre of the sheet a hole 1·5 cm. diameter was drilled to enable the reagents to be pipetted into the cups.

For use, this cover was either sterilised by ultraviolet light or merely wiped with a clean dry duster. It was found quite sufficient to wipe the dust off the cover in this way to prevent contamination, because the electrostatic charge produced attracts dust particles up to 1 cm. away from the charged surface. Vigorous rubbing must be avoided as the charge then induced is so strong as to pull drops off the end of the pipette, often on to the sides of the hole.

The cover proved invaluable in several ways besides preventing contamination. It provided a useful rest for steadying the hand during pipetting and in complicated experiments was extremely useful as a marker to indicate, by the position of the hole, the last cup inoculated.

To inoculate a tray the sheet of glass was removed and placed on a suitable sterile holder and the Perspex cover slid over the tray. After adding the reagents through the central hole (Fig. 1), taking care to avoid touching the sides, the cover was removed and the glass sheet replaced.

After use, trays were decontaminated by immersion in 10 per cent HCl overnight, rinsed in tap water and cleaned with a small brush— a rotating dental brush is particularly effective. After rinsing several times in distilled water the trays were dried, face down, at 37° and re-sterilised by exposure to ultraviolet light for a minimum of 30 min., during which time each tray was turned several times to ensure complete exposure. A sterile glass sheet was added and held in place with a Sellotape strip round the edge during storage.

Strains

All the strains used were freeze-dried after careful antigenic examination. The Vi strains were subcultivated daily according to the recommendations of Felix (1938) and Felix and Pitt (1951) in order to maintain them in a fully Vi state. Other strains were stored at 0–4° and subcultivated as required. At intervals lyophilised cultures were reconstituted to check those in use. The strains used were kindly provided from the National Collection of Type Cultures and included *Salm. typhi* 0901 (NCTC 5759), Ty2 (NCTC 4446), " Mrs S," an R variant (NCTC 8396) and a *Ballerup* strain (NCTC 6021).

Serum

Rabbit antisera were prepared by the intravenous inoculation of adult rabbits of approximately 3 kg. Each rabbit received a minimum course of 0·25, 0·5, 1·0, 1·0, and 2·0 ml. on

EXPLANATION OF PLATE

FIG. 1.—The Perspex cover in use during the preparation of a titration.

FIG. 2.—A chess board titration of antibody (O-antiserum) against antigen (*Salm. typhi* 0901).

Reading across: serum dilutions at 0·5 log. intervals, from 10^{-1} (columns 1 and 2) to 10^{-5} (column 10).

Reading down: antigen dilutions (10-fold) from undiluted peptone water culture of 0901 (top line) to 10^{-7} dilution (bottom line).

Serum control is column 1 (extreme left) which did not receive any complement. All the other cups received 1/3 dilution of absorbed guinea-pig complement. All cups received a 0·02 per cent solution of 2,3,5,-triphenyltetrazolium chloride.

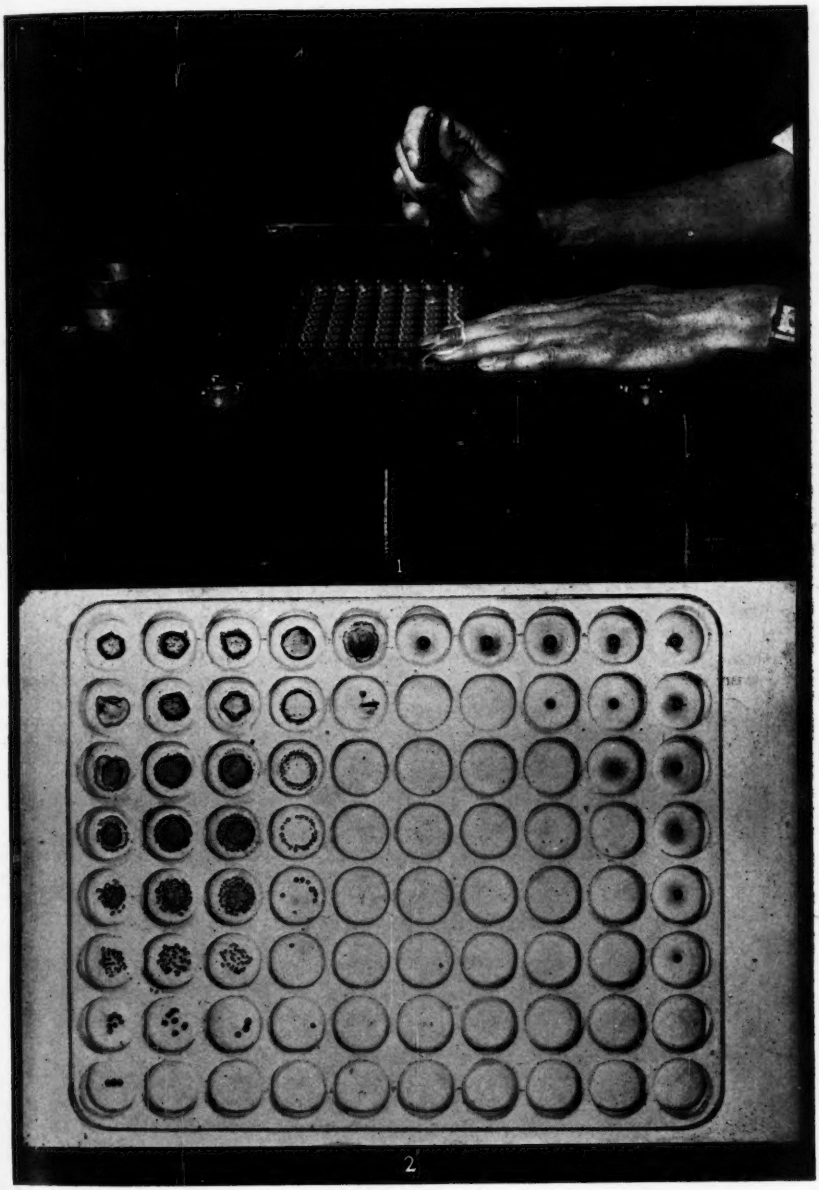
Note:

(*a*) The zone of 100 per cent killing of the inocula.

(*b*) The ease of reading with the formazan-coloured organisms.

(*c*) The clearly shown agglutination in the serum excess zone on the left. The large inocula at the top give the agglutination patterns described by Felix whilst the small inocula at the bottom give agglutinates derived from single cells—counts of these are identical with Miles and Misra counts performed simultaneously on the same suspensions.

(*d*) The 10^{-6} antigen dilution (next to bottom line) is killed by the 10^{-5} serum dilution and an end-point is therefore not shown. This is the line selected for single line titrations.



Nagington.

alternate days, of bacterial suspension with an opacity of 3000×10^6 organisms/ml. When the agglutination titre was considered adequate they were bled from an ear vein 7 days after the last inoculation. Bacterial suspensions used as vaccines were killed by exposure to 100° for 30 min. except in the case of Vi strains which were exposed to 50 per cent ethanol at 37° for 20 hr. (Kauffmann, 1951; Felix, 1952) and the Ballerup strain which was used as a living vaccine.

No serum preservatives could be permitted and the prevention of contamination was therefore essential. Each rabbit was bled as aseptically as possible from a marginal ear vein and the blood allowed to stand at 0–4° overnight before separation of the serum. The serum was stored at −70° until required and only then was it inactivated. Before use frozen serum was allowed to thaw gradually and diluted in sterile normal saline, usually 1/10, before inactivation at 56° for 30 min. Dilution before inactivation destroys non-specific bactericidal action (Mackie and Finkelstein, 1932). Serum properdin is completely destroyed at this temperature (Pillemer *et al.*, 1954).

Serum dilutions were prepared in sterile normal saline and storage of the diluted serum was at 0–4°. Metal-capped glass tubes were found most suitable for rapid manipulation.

Complement

Guinea-pig complement was obtained from pooled serum prepared by bleeding groups of 20–30 young animals by cardiac puncture and taking up to 5 ml. from each. After separation of the clot at 0–4° overnight in sterile 150 ml. conical glass flasks the serum was removed and cleared by centrifugation. The normal antibody in the guinea-pig serum was removed by two absorptions each of 1 hr. duration. *Salm. typhi* 0901 was used for the absorption of O antibody, *Salm. typhi* Ty2 or Ty6S for Vi antibody, and *Salm. typhi* "Mrs S" (R variant) for R antibody. In each case the whole of the harvest from an 18 hr. culture on thick tryptic digest agar in a Roux bottle was used to absorb 10 ml. of guinea-pig serum, *i.e.*, 1/2 Roux per absorption. The O and R strains were killed by 100° for 30 min. and the Vi strains by exposure to 50 per cent ethanol at 37° for 20 hr. All absorbing suspensions were prepared immediately before use and washed at least 3 times in sterile saline to keep soluble antigen to a minimum. After the final absorption the serum was completely cleared by centrifugation and immediately distributed in 1 and 2 ml. sterile glass ampoules which were then stored at − 70°. The whole operation up to the preparation of the ampoules was performed at 0–4°.

Tetrazolium solution

It was found most convenient to prepare the solution of 2,3,5,-triphenyltetrazolium chloride (T.T.C.) in bulk and to distribute it in small quantities sufficient for a single titration. Chemically clean glassware is essential. After it was found that there are sufficient reducing substances in digest broth to produce appreciable reduction of T.T.C. to red formazan during autoclaving, 1·0 per cent T.T.C. was prepared in normal saline, distributed in 1·0 ml. volumes in bijou bottles and sterilised immediately by 15 lb. pressure for 15 min. The sterile solution will keep indefinitely if stored away from the light at 0–4°. For use, the 1·0 per cent solution was added to digest broth immediately before it was required.

The lowest concentration of T.T.C. shown to produce any killing of the *Salm. typhi* strains used was 0·125–0·062 per cent. In practice it was found that an adequate red colour was produced and no killing was observed after the addition of a 0·02 per cent solution, which gave a final concentration of 0·016 per cent.

Method

To titrate the three variables, antigen (bacterial cell), antibody (immune rabbit serum) and complement (absorbed guinea-pig serum), use was made of a series of replicate chess board titrations of the kind described by Dean and Webb (1926). For this a number of Perspex haemagglutination trays were used. On each tray was prepared the same titration of antigen dilutions, consisting of inocula of different sizes, against serum dilutions. Complement was titrated by adding a different dilution of absorbed guinea-pig serum to each tray.

A series of six 10-fold dilutions of an 18 hr. peptone water culture of *Salm. typhi* was prepared in half-strength tryptic digest broth. On each of a series of trays, starting with the 10^{-6} dilution, an end row of cups was inoculated with 0·02 ml. per cup (one drop from a

Morse gauge 57 dropping pipette). The next row then received 0·02 ml. of the next dilution (10^{-5}) and so on until 7 rows of cups had been inoculated. To estimate the size of the inoculum surface viable counts were prepared by the method of Miles and Misra (1938) from the 10^{-6} dilution.

To the tray was then added in a similar fashion, but at right angles, 0·02 ml. per cup of each of a series of dilutions of inactivated unabsorbed immune rabbit serum in sterile normal saline. It was found convenient to use a dilution series composed of 2 superimposed sets of 10-fold dilutions which gave an interval of approximately 0·5 log. unit. No attempt to mix the reagents was made. When each tray was complete it was incubated at 37° for 1 hr. to sensitise the cells.

At the end of this time 0·02 ml. of a freshly prepared dilution of complement in sterile normal saline was added so that all the cups of one tray received the same dilution of complement. Incubation was continued for a further 1 hr. during which the bactericidal action took place.

Then to all cups was added 0·2 ml. of 0·02 per cent T.T.C. solution in 1/2 strength digest broth. Incubation was continued for a further 18 hr. and the results read by inspection. The absence of killing of all the cells of a particular inoculum was shown by the presence of a deposit or agglutination pattern of growing cells stained bright red by the formazan produced by the reduction of the T.T.C. Reading was facilitated by raising the tray a few cm. above a sheet of white paper by means of a sheet of glass. A specimen result is illustrated in Fig. 2.

Controls

Control cups consisting of several of the highest concentrations of serum used and the smallest inoculum, in the absence of complement, are required in order to ensure that there is no killing as the result of incomplete inactivation. It is also necessary to include a complement control with no serum to make sure that absorption has been complete and that complement alone is not lethal. T.T.C. controls are not necessary unless a strain is being examined for the first time. Sterility controls are omitted, since contaminants are obvious from the appearances they produce.

RESULTS

If the size of the inoculum is expressed as a dilution it can be used in the conventional manner to indicate the quantity of antigen. The dilution of rabbit antiserum can be used to indicate the quantity of antibody and the dilution of guinea-pig serum the quantity of complement.

By recording the greatest dilutions of antibody and complement able to cause killing of all the cells introduced in each inoculum it is possible to plot contour lines of each of the three variables. These are similar in some ways to the contours described by Hoyle (1945) in influenza complement fixation.

The following remarks appear to apply equally well to the action of both O and Vi antibodies. The action of O antibody is based on the effect of anti-0901 sera on *Salm. typhi* 0901 and Ty2. That of Vi antibody is of anti-Ty2 and *Ballerup* sera on strains Ty2 and Watson. The action of R antibody presents a somewhat different picture and will be returned to later.

Antigen contours

When the dilutions of antibody and complement which produce 100 per cent killing of each inoculum are plotted a series of antigen contour lines is produced (Fig. 3). Each contour line forms the boundary of an area which is the zone of complete killing and this area diminishes progressively with increase in inoculum size.

Each antigen contour has an optimum point which represents the dilution of antibody requiring the least amount of complement to kill that inoculum. When

these optimal points are joined together a curve of optimum antibody dilution is produced.

Each contour is bounded by a pre-zone or prozone of incomplete bactericidal action in the region of antibody excess, *i.e.*, the Neisser-Wechsberg zone. On the opposite side of the curve is the post-zone of less than 100 per cent killing where there is insufficient antibody. There is a crowding together of the contours

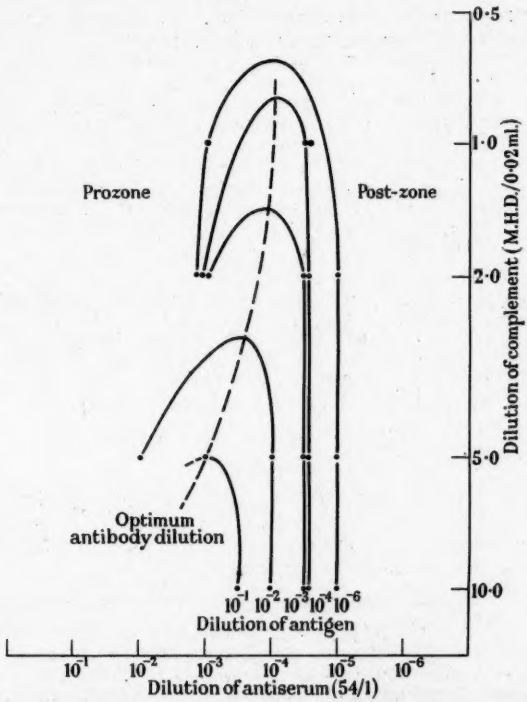


FIG. 3.—Antigen contours of 100 per cent killing of inocula of *Salm. typhi* 0901 by O-antiserum (54/1) and absorbed guinea-pig complement. All the dilutions are the initial dilutions, *i.e.*, before admixture with the other reagents. In constructing the diagram use has been made of interpolation between cups showing complete and less than complete killing of the inoculum. An antigen dilution of 10^{-6} gives a count of approximately 10 viable organisms in 0·02 ml.

on the right which indicates a rapid diminution of the effectiveness of the antibody with increasing dilution. The point of limiting antibody dilution is rapidly reached, often with the fusion of several contours.

Occasionally there is a part of the antigen contour which appears to represent inhibition by excess complement. This is seen in the higher antigen dilutions, *i.e.*, the smallest inocula, when there is a small prozone in the region of concentrated complement. It lies on the line of limiting antibody dilution for the particular inoculum. More usually the antigen contour flattens on the line of limiting anti-

body dilution (Fig. 3). It would appear that this indentation with concentrated guinea-pig serum is not necessarily due to excess of complement but may be due to soluble antigen introduced during the absorptions.

Complement contours

When antigen dilutions are plotted against antibody dilutions a series of complement contours is obtained (Fig. 4). The general appearance resembles that of the antigen contours, with a progressive increase of the area of complete killing as the quantity of complement is increased.

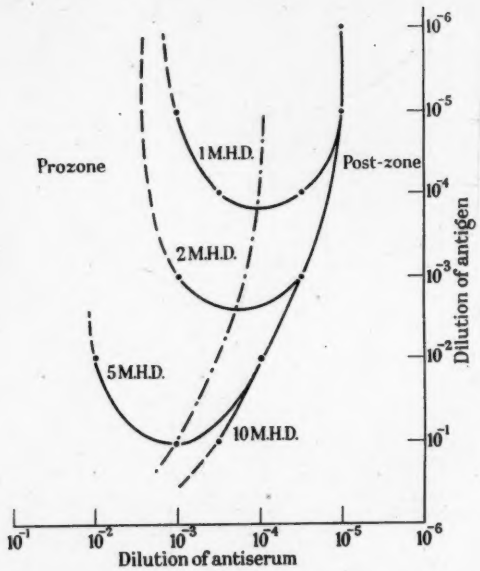


FIG. 4.—Complement contours of the titration shown in Fig. 3. Each contour encloses the zone of 100 per cent killing produced by the dilution of complement shown (M.H.D./0·02 ml.). All dilutions are the initial dilutions of the reagents. The broken central line joins the maximum effective complement dilutions for each inoculum. It is a projection of the optimum antibody dilution curve of Fig. 3.

Each contour has a point of maximum complete killing representing the largest inoculum completely killed by that dilution of complement. If these points are joined a curve is obtained which is the curve of maximum complement dilution capable of killing each inoculum. It is the curve of optimum antibody dilutions seen in different perspective. Increasing the concentration of complement increases the extent of killing, especially in the zone of antibody excess or prozone, on the left in Fig. 4. To the right of the zone of complete killing the effect is less marked, because the killing zone is limited by the limiting antibody dilution. As the amount of complement increases so the curve in the zone of dilute antibody tends to become a straight line and ultimately to coincide with the line of limiting antibody. Conversely, reduction in the amount of complement available produces

a progressive diminution of the killing zone until it retreats along the curve of optimum antibody concentration towards a point representing the smallest theoretical inoculum, before disappearing entirely.

Antibody contours

On first examination the graph of antibody contours (Fig. 5) appears to be very different from those of antigen and complement. To assist in the conception of these contours, it is useful to be able to visualise the picture of bacteriocidal action as a whole. There are three variables and from them a three-dimensional model can therefore be constructed. This has been drawn in Fig. 6. It will be seen that

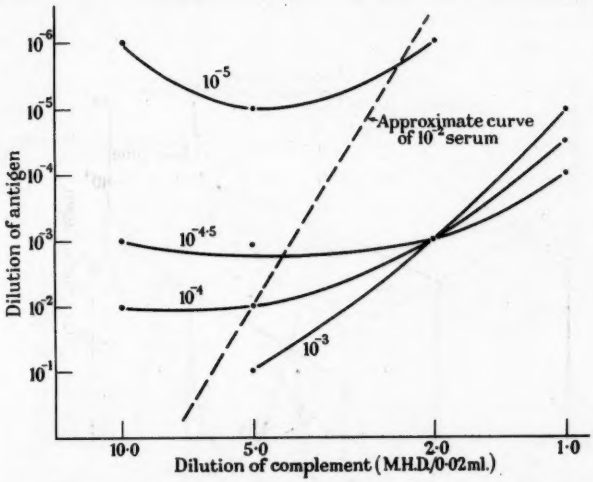


FIG. 5.—Antibody contours of the same titration of *Salm. typhi* 0901 and O-antiserum (54/1) shown in Fig. 3 and 4.

bactericidal action can be represented by a solid figure in the shape of a section of a boat. This figure encloses all the combinations of antigen, antibody and complement which result in 100 per cent killing of the inocula which comprise the antigen dilutions.

Continuing the analogy with part of a boat it will be seen that the keel is the line of optimum antibody dilutions and, at the tip of the bow, this reaches the point of maximum complement dilution. It will be seen that the antibody contours differ from those already described because they are the limits of vertical longitudinal sections of such a volume.

Consideration of Fig. 5 shows that, as the antibody excess prozone is left behind, increasing dilution of antibody produces a greater extent of killing and more dilute complement suffices. This continues until a point is reached after which further dilution of the antibody reduces its efficacy until it is eventually extinguished. This is due to optimal proportions of antibody to complement operating between the antibody excess prozone and the point at which there is insufficient antibody to provide adequate sensitisation for 100 per cent killing.

In Fig. 5 there is also shown a complement excess prozone with weak serum (10^{-5}). This prozone is especially common in titrations with R antibody and R strains of *Salm. typhi*.

The influence of the normal bactericidal antibody in guinea-pig serum

In the foregoing experiments the complement used had been absorbed with the test strain in order to remove any normal bactericidal activity. Guinea-pig serum often contains natural antibodies in sufficient quantity to alter the picture of the action of an immune serum. This effect is shown in Fig. 7, in which are recorded the antigen contours of the titration of 0901 and the same immune rabbit serum previously used, but with unabsorbed guinea-pig serum as the source

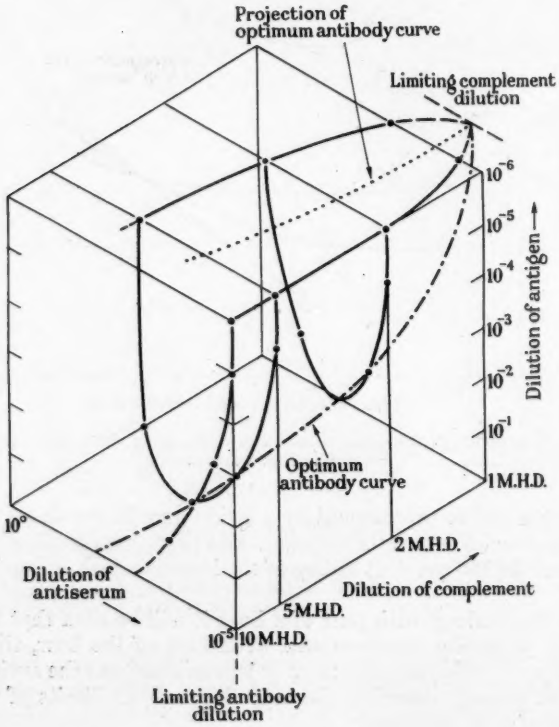


FIG. 6.—Three-dimensional representation of bactericidal action of O-antiserum (54/1) and absorbed guinea-pig complement on *Salm. typhi* 0901. The diagram is produced from Fig. 3, 4 and 5 and for simplicity only a selection of contours have been drawn. The horizontal contour is the 10^{-6} antigen contour, the vertical contours are complement contours. The limiting dilutions of complement and antibody are shown. The limiting or maximum dilution of antigen is the 10^{-6} dilution, *i.e.*, an inoculum of approximately 10 viable organisms, which is the smallest practicable inoculum. The optimum antibody curve is capable of projection in two directions; that in the 10^{-6} antigen dilution plane is shown (dotted line) but that in the 10 M.H.D. complement plane (see Fig. 4) has been omitted.

of complement. The 10^{-6} antigen contour of the previous titration, in which absorbed complement was used, has been superimposed for comparison.

Omitting absorption produces a considerable increase in the extent of killing. The unabsorbed complement has an apparent maximum effective dilution in the region of 1/100 compared with 1/20 for absorbed complement. In both sets of titrations the complement used was of the same haemolytic titre (400 M.H.D./ml.).

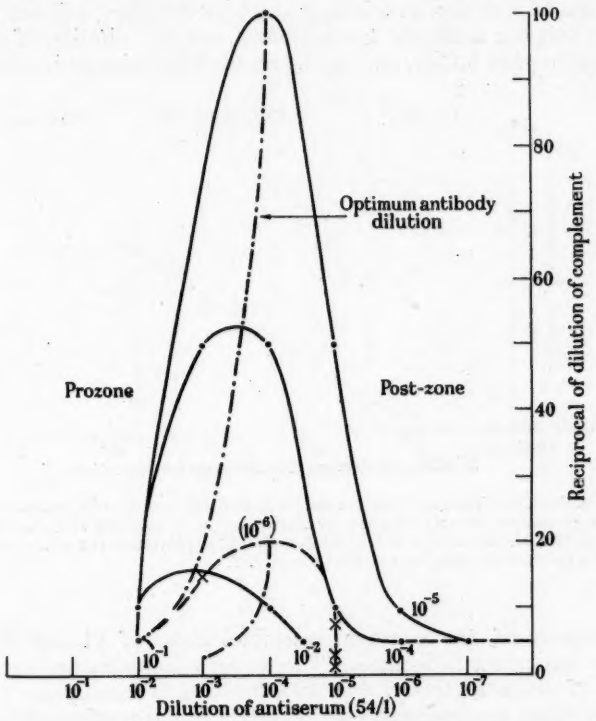


FIG. 7.—Antigen contours of the bactericidal action of O-antiserum (54/1) on *Salm. typhi* 0901 mediated by *unabsorbed* guinea-pig complement of the same haemolytic titre (400 M.H.D./ml.) as the absorbed complement previously used. For comparison the 10^{-6} antigen contour of the titration with absorbed complement (Fig. 3) is shown by a broken line. Note the greater extent of 100 per cent killing, especially in the post-zone of dilute antibody, and the "tailing-off" effect due to killing by concentrated complement without the addition of antibody.

The natural antibody makes a considerable difference to the dilute antibody region. The limiting antibody dilution is obliterated due to a "tailing-off" effect produced by the presence in the guinea-pig serum of sufficient antibody, and perhaps other bactericidal substances, in dilutions of less than 1/10, so that small inocula are killed in the absence of added immune antibody.

The presence of the small amount of natural antibody does not appreciably affect the prozone, probably because the quantity of such antibody is so small

in comparison with the immune antibody. It is interesting that the optimum antibody dilution at the point of maximum complement dilution is the same with both absorbed and unabsorbed complement, *i.e.*, 10^{-4}.

The relationship between antibody and complement

If the curve of optimum antibody dilutions is plotted on a logarithmic scale of antibody against complement dilutions a straight line is obtained (Fig. 8). Over the range of 100 per cent killing there is therefore a linear relationship between the optimum antibody concentration and the dilution of complement. This linear relationship holds down to the smallest practicable inoculum, 10^{-6}.

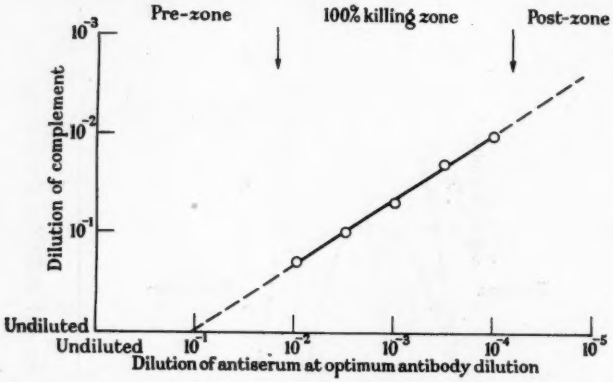


FIG. 8.—The relationship between optimum antibody dilution and the dilution of complement (unabsorbed guinea-pig serum) required by that dilution to produce 100 per cent killing. The points plotted are taken from the optimum antibody dilution curve of the O-antiserum titration with *unabsorbed* complement shown in Fig. 7.

R antibody

The appearance of the contours resembles those of Vi and O antibodies, but there are important differences. The antigen contours are usually convex in the region of dilute antibody, due to a prozone of incomplete killing in the presence of the most concentrated complement. This is particularly marked with increasing inoculum size. There is therefore an optimum complement dilution with high antibody dilutions.

Complement is apparently more effective with R antibody. A particular batch of complement is able to mediate 100 per cent killing of R strains with R antibody at much greater dilution than in the case of O and Vi strains and their respective antibodies. But the area of 100 per cent killing is smaller, mainly because of a wider Neisser-Wechsberg zone which produces a much narrower band of killing.

A method for the bactericidal titration of antibody

From these results it can be seen that there are three possible ways of titrating antibody by means of its bactericidal action: (1) The maximum or limiting antibody dilution can be measured. This is the greatest dilution of serum which is capable of killing 100 per cent of the smallest practicable inoculum in the presence

of excess or, if there is zoning, optimum complement. (2) By determining the relation between the optimum and limiting antibody dilutions and then by finding the optimum dilution of the serum at a convenient complement level, say the minimum or limiting complement concentration, the limiting antibody dilution may be calculated. (3) Finally, the strength of a serum might be expressed simply as the optimum serum dilution at the point of limiting complement dilution.

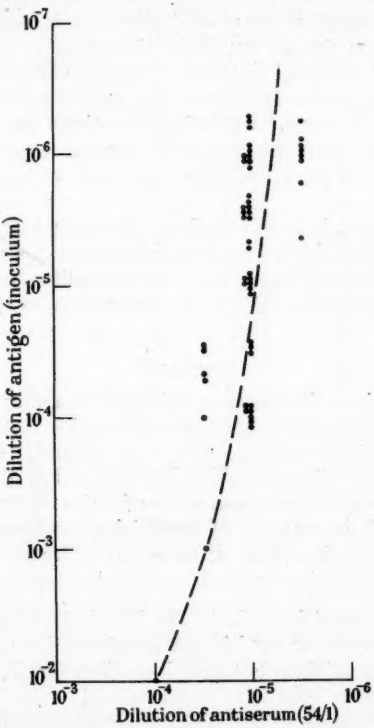


FIG. 9.—Complement contour from 57 different titrations of O-antiserum (54/1) against *Salm. typhi* 0901 when 5 M.H.D./0·02 ml. of different batches of absorbed guinea-pig complement was used. Each point shown represents the estimated inoculum size derived from a Miles and Misra count. There is clearly no marked variation in maximum antibody dilution to be expected over the range of size of inocula from the 10^{-6} antigen dilution ($8{\cdot}45 \pm 6{\cdot}5$ viable organisms for $P = 0{\cdot}05$).

In practice the first method is the most economical of materials and alone permits titration by means of a single row of serum dilutions. A 10^{-6} dilution of an 18 hr. peptone water culture of *Salm. typhi* yields a mean count of approximately 10 organisms in 0·02 ml. In a large series of viable counts on different strains the arithmetic mean counts of individual inocula all fell between 6·04 (Ty6S) and 11·4 (Ty2) organisms per 0·02 ml. From a series of more than 500 observations it appears that the probability of a cup receiving no viable organisms from such a 10^{-6} dilution of any strain of *Salm. typhi* would not be greater than 0·002.

The method of titration is exactly the same as already described for the chess board titrations but only a single row of serum dilutions is required. To each cup is added 0·02 ml. of a 10^{-6} dilution of inoculum, 0·02 ml. of serum dilution and 0·02 ml. of absorbed complement diluted to give either 5 M.H.D. in 0·02 ml. or the optimum dilution for the test strain.

Such a titration is part of a complement contour. It should give a reliable estimate of bactericidal antibody provided that the slope of the contour is not too steep over the distribution range of individual inocula. In Fig. 9 is shown the complement contour for 57 separate titrations of a single serum against 0901 using 5 M.H.D. of different batches of complement. It illustrates what was found in practice, that the size of the inoculum has little effect on the titre obtained when a small inoculum is used. Variation is mainly due to such factors as the difficulty in reproducing serum dilutions with sufficient accuracy. The Percentage Law of Andrewes and Elford (1933) does not appear to apply to this region of small inocula.

A more accurate result is obtained from the titration of two different sets of dilutions of a serum than from two replicate titrations of one set of dilutions. The addition of 0·063 per cent $MgCl_2.6H_2O$ to the saline diluent used for the serum and complement would probably be an improvement (Muschel and Treffers, 1956).

SUMMARY

The bactericidal action of serum is considered as a simplified system consisting of three components; antigen (the bacterial cell), antibody (immune rabbit serum) and complement (absorbed guinea-pig serum). By fixing two of these variables the third can be titrated. The bactericidal action of immune antibody can be titrated in at least three ways. One of these is by finding the maximum dilution of serum which is capable of sensitising an inoculum of approximately 10 viable cells sufficiently for them all to be killed by the addition of guinea-pig complement. A simple practical method of titration based on this is suggested.

Grateful acknowledgment is made to Mr. D. S. Miller for technical assistance, to Professor E. T. C. Spooner for his encouragement and advice, to Mr. W. T. Bush for the preparation of the photographs and to Dr. J. A. Boycott for reading the manuscript.

REFERENCES

Adler, F. L.—(1953) *J. Immunol.*, **70**, 69.
Andrewes, C. H. and Elford, W. J.—(1933) *Brit. J. exp. Path.*, **14**, 367.
Dean, H. R. and Webb, R. A.—(1926) *J. Path. Bact.*, **29**, 473.
Felix, A.—(1938) *J. Hyg., Camb.*, **38**, 757.—(1952) *Ibid.*, **50**, 515.
Idem and Pitt, R. M.—(1951) *Ibid.*, **49**, 92.
Hoyle, L.—(1945) *Ibid.*, **44**, 170.
Kauffmann, F.—(1951) *Enterobacteriaceae*. Copenhagen (Einar Munksgaard).
Mackie, T. J. and Finkelstein, M. H.—(1932) *J. Hyg., Camb.*, **32**, 1.
Miles, A. A. and Misra, S. S.—(1938) *Ibid.*, **38**, 732.
Muschel, L. H. and Treffers, H. P. (1956) *J. Immunol.*, **76**, 1.
Pillemer, L., Blum, Livia, Lepow, J. H., Ross, O. A., Todd, E. W. and Wardlaw, A. C.—(1954) *Science*, **120**, 279.
Pulvertaft, R. J. V.—(1930) *Lancet*, *i*, 615.
Watson, K. C.—(1955) *J. Lab. clin. Med.*, **46**, 128.

THE SENSITIVITY OF *SALMONELLA TYPHI* TO THE BACTERICIDAL ACTION OF ANTIBODY

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THE first demonstration of the ability of antibody to sensitise *Salmonella typhi* to the lethal action of complement was made by Felix and Olitski (1926) when investigating O antibody. Later it was found that rough (R) strains were susceptible to R antibody (Adler, 1953*b*) and recently it has been shown that Vi antibody may also cause specific sensitisation (Muschel and Treffers, 1956). There is no evidence of similar activity on the part of H antibody. In the present work the activity of the bactericidal antibodies is considered in relation to the antigenic structure of the bacterial cell.

MATERIALS AND METHODS

The classical strains used were supplied by the National Collection of Type Cultures. They included *Salm. typhi* 0901 (NCTC 5759), Ty2 (NCTC 4446), "Watson" (NCTC 5761), Ty6S (NCTC 8383), ViI (NCTC 8222) and a completely rough variant of strain "Mrs S" (NCTC 8396). Several recently isolated strains, including T13 (T 5277), were from the Enteric Reference Laboratory, Colindale. *Salm. ballerup* (NCTC 6021) was received from Dr. F. Kauffmann with the strain number K 107, 7851, and is assumed to be the original type strain. A rough strain of *Salmonella paratyphi* C (R30) was from the London School of Hygiene.

Except where otherwise stated unabsorbed immune rabbit antiserum was employed in the experiments to be described. The method of preparation of this and also absorbed guinea-pig serum, which was used as a source of complement, has already been described (Nagington, 1956).

Bactericidal titration was performed by the technique described in the preceding paper. In this method, inocula of about 10 viable organisms were sensitised by the addition of antiserum and then complement was added to produce killing. To facilitate the detection of survivors use was made of the ability of growing cells to reduce tetrazolium salt to a red formazan. The bactericidal titre of a serum is expressed as the greatest dilution of serum capable of producing sufficient sensitisation of the cells in the inoculum for them to be killed by the addition of adequate complement.

Agglutination tests and the maintenance of strains were according to the methods of Felix and Pitt (1951).

To compare the agglutinating and bactericidal titres of immune rabbit O and Vi antisera on strains of organisms containing both O and Vi antigens (O + Vi strains) the following method was based on that used by Felix and Pitt for the preparation of Table 2 in their 1951 paper. Cultures of *Salm. typhi* Ty2, "Watson" and a recently isolated wild strain, T 13, were subcultivated daily until judged to be in a state of maximum Vi development. A culture of 0901 was maintained fully smooth and subjected to identical treatment. Fresh 18 hr. cultures of all 4 strains were harvested in sterile saline and divided into 3 parts. One part was diluted in half-strength tryptic digest broth for use as the inoculum in bactericidal titrations. The second part was killed with 0·05 per cent formalin and the third killed

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by heating at 100° for 2 hr. Then the agglutinability of both killed suspensions by O and Vi antisera was measured and compared with the bactericidal activity of the same sera on the living organisms.

RESULTS

The Sensitivity of O + Vi Typhoid Strains

The O + Vi strains of *Salm. typhi* are the smooth virulent strains such as Ty2, " Watson " and the majority of naturally occurring strains. A set of titrations to illustrate their sensitivity to bactericidal action compared with their agglutinability is reproduced in the form of a histogram in the Figure, in which the bactericidal and agglutination titres of the O and Vi antisera against each suspension are expressed as a percentage of the possible maximum titre attainable.

The strains are arranged in the form of a series with increasing Vi development, from 0901 (smooth and completely devoid of Vi antigen) to Ty2 with the greatest amount of Vi. The development of O-inagglutinability with increasing amount of Vi antigen is well demonstrated. The effect described by Felix and Pitt (1934; 1951) of less than maximum Vi-agglutinability in forms with the greatest Vi development is also illustrated.

Bactericidal sensitivity can be seen to be parallel, but not coincident, with agglutinability. This might be expected as a result of the far greater sensitivity of bacteria to the bactericidal than to the agglutinating action of antibody. When Vi antigen has covered or in some other way affected the surface of the organism to reduce O-agglutinability, there will presumably still be ample O receptors left uncovered to enable O antibody to kill. According to Adler (1953*b*) the combination of antibody with one in a hundred receptors on the surface of 0901 is sufficient to enable complement to kill.

Strains possessing intermediate amounts of Vi antigen showed greatest sensitivity to Vi antibody killing, but Ty2 which, according to Felix and Pitt (1951) can be shown by quantitative agglutinin absorption tests and by its antigenicity to contain more Vi antigen than " Watson," was less sensitive both to agglutination (as shown by Felix and Pitt, 1951) and to killing. Felix and Pitt explain their observations by supposing that Ty2 contains an excess of Vi antigen, making it inagglutinable by the higher dilutions of Vi antibody. A similar explanation, amplified in the Discussion below, may serve to explain the low sensitivity of Ty2 to Vi antibody killing.

It is conceivable that an organism may develop such an amount of Vi antigen that Vi antibody may no longer be able to exert any bactericidal action at all. This may be the case in strains such as *Salm. ballerup* which was found resistant not only to typhoid Vi antibody but also to rabbit antiserum prepared against the homologous living organisms. This insensitivity of *Salm. ballerup* to the bactericidal action of Vi antiserum was noted also by Muschel and Treffers (1956).

The Sensitivity of R + Vi Strains

There are two classical strains of *Salm. typhi* in which the predominating antigens are R and Vi. Strain Ty6S (Felix and Petrie, 1938) is sometimes called a " pure " Vi variant (Felix and Pitt, 1951) or " Vi half-smooth form " (Scholtens 1937) because it is antigenically " rough ", that is, completely devoid of O antigen. Morphologically it does not appear to be rough and it is stable in 5 per cent saline.

Strain ViI (Bhatnagar, Speechly and Singh, 1938) is similar but contains a small amount of O antigen. This is insufficient to cause agglutination by O antiserum but gives rise to the production of O antibody when ViI is used as a vaccine (Felix, 1938). In many ways ViI behaves as intermediate between Ty6S and the O + Vi strains. It is convenient to consider the two strains ViI and Ty6S together since their major antigens are the same, apart from the presence of the trace of O in ViI.

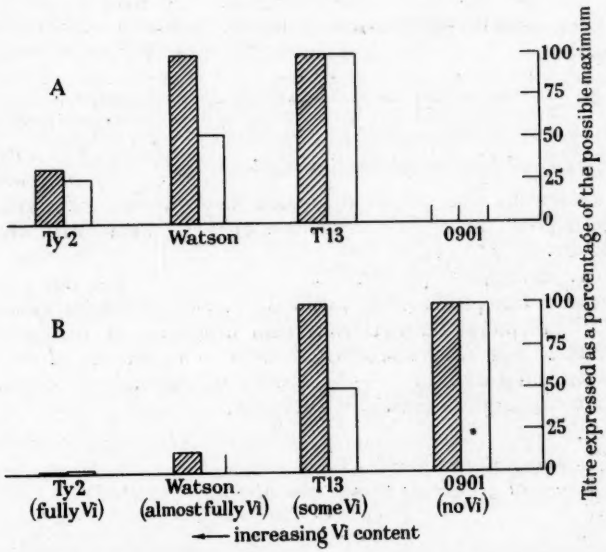

FIGURE.—Histogram of the agglutinability and bactericidal sensitivity of 0 + Vi strains of *Salm. typhi*. A. Vi antiserum (54/2). B. O antiserum (54/1). Titre expressed as a percentage of the possible maximum. Bactericidal action shown by hatched columns. Agglutination shown by white columns.

Vi antibody prepared from smooth vaccine strains

Two rabbit antisera (53/7 and 54/2) prepared against smooth fully Vi living strains of *Salm. ballerup* (NCTC 6021) were without effect on both Ty6S and ViI. Yet both sera contained abundant Vi antibody and this was fully bactericidal against O + Vi typhoid strains (Table I).

Immune antiserum prepared against a formolised suspension of Ty2 was equally ineffective against Ty6S and ViI. It has been shown that Vi antibody prepared from formolised Ty2 is "functionally inadequate" in passive-protection tests in mice (Felix and Bhatnagar, 1935; Felix, 1938; 1952) and also in complement fixation tests (Henderson, 1939), but there is no evidence that it is other than functionally adequate in its bactericidal action against the O + Vi strains Ty2 and "Watson" when the present method is used (Table I).

From these results it is thought probable that Vi antibody which is derived from smooth vaccine strains is without action on R + Vi strains of *Salm. typhi*.

TABLE I.—*Insensitivity of Strains Ty6S and ViI to Vi Antibody from Smooth Vaccine Strains*

Rabbit serum.	Vaccine.	Maximum bactericidal titre recorded against the following test strains. Ty2 (O + Vi).	0901 (O).	"Mrs S" (R).	Ty6S.	ViI.
54/2	*Salm. ballerup* (living)	100,000	100	100	30	<10
53/7	" "	50,000	300	<10	<10	<10
53/4	Ty2 (formolised)	(10,000)*	300,000	<30	100	<30
53/4 (after absorption with 0901)	" "	3,000	<30	<30	<30	<30

* Part of this titre is due to killing by the O antibody.
Note: titres of 100 or less are probably due to normal antibodies.

Vi antibody prepared from rough vaccine strains

Rabbit antiserum was prepared against two vaccines of Ty6S—alcoholised and heat-killed (100° for 2½ hr.), a vaccine of ViI which had been treated with N-HCl for 20 hr. at 37° (Felix, 1952) and an alcoholised vaccine of the rough strain of *Salm. paratyphi* C (R 30).

All of these strains produced Vi antibody which was measurable by agglutination. This Vi antibody differed from that produced by antigenically smooth strains in that it was fully bactericidal to R + Vi strains of *Salm. typhi*. It was fully bactericidal also to O + Vi strains of *Salm. typhi* (Table II) and so could readily be distinguished from R antibody.

TABLE II.—*Sensitivity of Strains Ty6S and ViI to Vi Antibody from a Rough Vaccine Strain and also to R Antibody*

	Maximum bactericidal titre recorded against the following test strains. "Mrs S" (R).	Ty2 (Vi).	Ty6S.	ViI.
Immune rabbit serum 54/13 prepared against an alcoholised vaccine of *Salm. paratyphi* C rough strain (R30)—				
Unabsorbed	3,000	30,000	30,000	30,000
Absorbed twice with an alcohol-killed *Salm. ballerup* suspension to remove Vi antibody	3,000	<10	1,000	1,000
Absorbed twice with *Salm. ballerup* and then twice with heat-killed "Mrs S." to remove Vi and R antibodies	<10	<10	<10	<10
Absorbed twice with a heat-killed suspension of "Mrs S" to remove R antibody	<10	10,000	10,000	30,000
Immune rabbit serum 54/8 prepared against a heat-killed vaccine of *Salm. typhi* "Mrs S"	3,000	<10	1,000	1,000

Serum 54/13 (unabsorbed) had a standard Vi agglutination titre of 160 measured against the Standards Laboratory agglutinating suspension. A bactericidal titre of 100 against *Salm. typhi* 0901 and an agglutination titre of less that 40 is taken to indicate only a trace of normal O antibody.

When antigenically smooth Vi strains of *Salm. typhi* and *Salm. ballerup* were used to absorb these sera it was found that all Vi agglutinins were removed and also all the Vi antibody bactericidal to O + Vi strains. The results of absorptions with *Salm. ballerup* are illustrated in Table II. The bactericidal activity against

ViI and Ty6S was almost completely removed. On some occasions the activity of the absorbed serum towards Ty6S did not show the same drop in activity as against ViI. Assuming that there are only the two antigens Vi and R present in Ty6S the remaining factor was therefore either unabsorbed Vi or R antibody. Since absorption with a suspension of the pure R strain "Mrs. S" completely removed this residual activity the factor was almost certainly R antibody.

R antibody

The pure R antibody serum produced by a vaccine of *Salm. typhi* "Mrs. S." is bactericidal to both Ty6S and ViI (Table II).

O antibody

There is no evidence that O antibody can kill either ViI or Ty6S, although it is conceivable that under the appropriate conditions some action on ViI might occur.

It is concluded that both ViI and Ty6S are sensitive to the action of Vi antibody produced in rabbits against antigenically rough Vi strains, *i.e.*, strains which are capable also of stimulating the production of R antibody. Ty6S is very sensitive to the R antibody produced both by these strains and purely R strains. ViI tends to be less sensitive to R antibody but is still highly sensitive to it. This is what might be expected in a strain in which there is still a small amount of O antigen.

According to Kennedy and Stuart (1950) R-agglutinability in R + Vi strains can be masked by the Vi antigen. This effect may be reflected by variation in the sensitivity of the strains to the bactericidal action of R antibody. If Vi antigen development is sufficiently marked it is reasonable to assume that some hindrance will be produced to the accessibility of the R antigen to its antibody, which will reduce the bactericidal efficacy of the R antibody in the same way that Vi antigen reduces the agglutinability and bactericidal power of O antibody in O + Vi strains.

R antibody

The discovery of the sensitivity of the R + Vi strains to the bactericidal action of R antibody as well as to the Vi antibody produced by rough vaccine strains prompted further investigation of the antigenicity of the R substance. There is considerable confusion in the literature and much of this appears to be due to the similarity of the properties of the R and Vi antibodies (Kennedy and Stuart, 1950).

Table III shows the results of the bactericidal titration of immune rabbit sera produced against various vaccines. The bactericidal method is more sensitive than agglutination methods of measuring antibody and it is of especial value in the case of R antibody where agglutination methods are of limited value. Strains 0901 and "Mrs. S." are sensitive only to O and R antibody respectively, but Ty2 is usually sensitive to O as well as Vi antibody. Unfortunately, no typhoid strain has yet been found which is sensitive to the bactericidal action of Vi antibody alone. Consequently the titres against Ty2 in the Table are of unabsorbed sera for those in which there is no appreciable O antibody and after absorption with 0901 for those sera with appreciable O antibody.

TABLE III.—*Antigenicity of R in Relation to the Amount of O and Vi Antigen Present in Various Vaccine Strains.*

Immune antibodies present in the antiserum.			Immune antiserum. Rabbit.	Nature of vaccine used.	Maximum bactericidal titres recorded against the following test strains. Ty2 (Vi).	0901 (O).	" Mrs S " (R).
Vi	—	—	54/2	*Salm. ballerup* (live)	100,000	100	100
Vi	—	—	53/7	„ „ „	50,000	300	<10
—	O	—	54/1	0901 (alcoholised)	(3,000)	300,000	10
Vi	O	—	53/4	Ty2 (formolised)	3,000*	300,000	<30
Vi	O	R	53/5	ViI (alcoholised)	10,000*	10,000	1,000
(Vi)	O	R	53/3	„ (heated)	(300)	1,000	1,000
Vi	—	R	53/1	„ (HCl-treated)	3,000*	300	3,000
Vi	—	R	54/11	Ty6S (alcoholised)	100,000	300	1,000
Vi	—	R	54/12	„ (heated)	10,000	100	1,000
Vi	—	R	54/13	*Salm. paratyphi* C (R30—alcoholised)	30,000	100	3,000
—	—	R	54/6	*Salm. paratyphi* C (R30—heated)	(300)	300	1,000
—	—	R	54/8	Mrs S (heated)	<10	100	3,000

Titres marked with an asterisk are following absorption with 0901 to remove all the O antibody. The Ty2 titres shown in brackets are partly or entirely due to O antibody. All titres are the reciprocal of the initial dilution of serum, *i.e.*, before admixture with any of the other reagents. Normal antibody may give titres up to 300.

It will be seen that after inoculation with fully O strains no R antibody is produced (the course of injections was of not less than 5 injections on alternate days, which made a total dose of approximately 15×10^9 cells). This would perhaps seem to be a restatement of an obvious and well known fact. But the point to which it is wished to draw attention is that a progressive loss of O antigen leads to an equally progressive increase in the antigenicity of the R substance; R antibody is produced and this process is quite independent of the presence of the Vi antigen. Hence the use of " pure Vi " strains for the production of sera is attended by the production of considerable R antibody and this antibody can react with such " pure Vi " strains in a closely similar manner to Vi antibody.

This may explain the agglutination by normal sera of a ViI suspension used by Brower (1944) who noted that the effect was due to an antibody other than Vi. Wheeler and Stuart (1946) found a " Vi-like " antigen in several coliform and paracolon strains, and Kennedy and Stuart (1950) christened it the ' Er ' antigen, *i.e.*, *Enterobacteriaceae* rough. It was found in many organisms known to possess Vi antigen and they found that sera thought to contain only Vi antibody may contain also ' Er ' antibody.

If, for " Vi-like " and Er, we substitute R antibody the picture becomes clearer. A recent example of the occurrence of this antibody in " pure Vi " antiserum may be provided by the additional specificity of typhoid Vi antibody (produced against ViI extract) when compared with *Salm. ballerup* Vi antibody which was described by Chi-Yen Chu and Hoyt (1954). When Ty6S was substituted for ViI in the preparation of sera by the technique they described, a high-titre R serum was produced with so little Vi antibody that the method might well

be used for the preparation of R antiserum. It may be that other antigens which have been described, such as the X antigen of Topley and Ayrton (1924) would, on re-examination, be found to be R antigen.

The Bactericidal Sensitivity of the Classical Typhoid Strains

The classical strains of *Salm. typhi* are well defined stable types which are particularly useful in serological work. Their sensitivity to bactericidal antibody is shown in Table IV. Those which are sensitive to only a single antibody are

TABLE IV.—*Bactericidal Sensitivity of the Classical Strains of* Salm. typhi.

Strain.	Antigenic structure.				Antibodies which can exert a bactericidal action.				Remarks.
Ty2	H	Vi	O	—	—	Vi	O	—	Sensitive to all Vi antibodies. Insensitive to O antibody when fully Vi.
"Watson"	H	Vi	O	—	—	Vi	O	—	
H901	H	—	O	—	—	—	O	—	
0901	—	—	O	—	—	—	O	—	
ViI	H	Vi	O tr.	R	—	Vi	—	R	Sensitive only to the Vi antibody produced against R vaccines.
Ty6S	H	Vi	—	R	—	Vi	—	R	
"Mrs S" (rough variant)	H	—	—	R	—	—	—	R	

Table based on that of Felix and Pitt (1951), from which the production of R antibody was omitted.

straightforward (H901, 0901, "Mrs. S."). Others, which are sensitive to two antibodies (Ty2, "Watson") need greater care in their use as test strains, and control titrations must always be employed to determine their senitivity to each antibody on every occasion. The R + Vi strains (Ty6S, ViI) are the least suitable of the group on account of their peculiar sensitivities and, compared with the other strains, they also show a much greater degree of variation in susceptibility.

DISCUSSION

A culture of *Salm. typhi* on first isolation from a case of enteric fever may be considered to represent the nearest approach to full antigenic development *in vitro*. The surface of such organisms is almost always composed of Vi antigen. On continued subculture the Vi antigen is gradually lost unless certain conditions are observed. The O antigen then enters into the composition of the surface layer and with complete loss of Vi it forms the entire surface. Further "loss variation" (Kauffmann, 1951) with reduction in the quantity of O antigen is accompanied by the development of antigenicity by what is apparently an underlying substance, the R substance. This process produces a mixture of O and R antigens on the cell surface in the same way as during the loss of Vi it was composed of Vi and O. At the completion of the process the surface is probably again uniform, the organism completely rough and the surface composed of R antigen.

It is to be expected that most strains of *Salm. typhi* in the laboratory will have a surface layer composed of a mixture of two antigens. They can therefore be sensitive to the bactericidal action of two antibodies. If however only one bacteri-

cidal antibody is presented to such a cell then susceptibility to this will depend on the amount of that particular antigen, that is " susceptible " antigen, which is accessible to the antibody. In consequence there are at least three possible ways in which reduction or loss of sensitivity to bactericidal action may be expected to occur :

(*a*) Decrease in the actual quantity of susceptible antigen in the cell.

(*b*) Increase in the quantity of insusceptible antigen as in the case smooth O + Vi strains and probably also in R + Vi strains.

(*c*) Increase in the quantity of *susceptible* antigen.

This proposed third mechanism is analogous to the reduction in Vi-agglutinability when Vi antigen increases in quantity past a certain point, which is shown well with Ty2 and " Watson ". The mechanism is the same as in (*b*) but, instead of the coating of insusceptible antigen making inaccessible the susceptible antigen, in this case the increasing thickness of susceptible antigen itself acts as a barrier. The reduced sensitivity of Ty2 to the action of Vi antibody is considered to be an example of this effect. It may also provide an explanation for the insusceptibility of *Salm. ballerup* and some *Bacterium coli* strains to the bactericidal action of Vi antibody.

It is probable that the effect is only seen in Vi and similar antigens which can form a relatively thick envelope around the bacterial cell. It has been shown that in O-inagglutinable Ty2 there may be as much as ten times the quantity of Vi antigen that is present in O-agglutinable strains such as the American " 58 " strain (Webster, Sagin, Anderson, Breese, Freeman and Landy, 1954). The mechanism protecting the bacterial cell from the Vi antibody is probably not a simple one but may well consist of several parts. Antibody may be rendered harmless by combination with soluble Vi antigen and hapten at the periphery of the Vi envelope. It may be held away from the vital part of the cell purely by the thickness of the Vi layer and perhaps even be actively repelled by *Quelling* (Landy, 1954) when it attaches itself to the antigen. In each case the subsequent combination of complement with the antigen-antibody complex then takes place so far removed from the vital part of the cell wall that it is rendered impotent and the cell is undamaged. It is suggested that this is a possible way in which the invading typhoid bacillus is protected from the antibody defences of the host.

Another possible method by which the typhoid organism can be protected from the bactericidal action of antiserum was proposed by Adler (1953*a*, *b*) who suggested that R antibody could, by attachment to the cell, impede the action of O antibody on 0901. The demonstration of this effect depends on the simultaneous introduction of all three factors, antigen, antibody and complement. Competition for complement may therefore be a major factor. Unfortunately, even after alteration of the method and sequence of addition of the reagents, it has not been possible to elicit the effect by means of the present method of bactericidal titration.

A special problem is presented by the antigenically rough strains Ty6S and ViI. They are susceptible to killing by R antibody and also by Vi antibody, but apparently only homologous Vi antibody or that produced against rough vaccine strains. This would seem to provide evidence of the necessity for a particular fit of Vi antibody, determined by its formation against Vi antigen in close proximity to R antigen. This might provide a clue to the curious ability of a Vi-phage to lyse organisms which contain no Vi antigen which was reported

by Cherry, Davis and Edwards (1954). The Vi-phage may have been able to utilise R antigen receptors.

SUMMARY

The bactericidal action of O antibody on *Salm. typhi* can be blocked by Vi antigen covering the surface of the cell in the same way that Vi antigen can produce O-inagglutinability. This effect is thought also to occur with R antibody and R + Vi strains (Ty6S and ViI) where Vi antigen covers the R antigen. Such R + Vi strains are apparently sensitive only to homologous Vi antibody and not to that formed against smooth vaccine strains.

The sensitivity of the classical strains of *Salm. typhi* to bactericidal antibodies is presented.

A mechanism by means of which the bacterial cell may be protected against Vi antibody through the increased development of Vi antigen is discussed, as is the R antigen and the confusion its presence in Vi strains may cause.

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REFERENCES

Adler, F. L.—(1953*a*) *J. Immunol.*, **70**, 69.—(1953*b*) *Ibid.*, **70**, 79.
Bhatnagar, S. S., Speechly, C. G. J. and Singh, M.—(1938) *J. Hyg. Camb.*, **38**, 663.
Brower, W. H.—(1944) *Amer. J. Hyg.*, **40**, 154.
Cherry, W. B., Davis, B. R. and Edwards, P. R.—(1954) *Science*, **120**, 309.
Chi-Yen Chu, D. and Hoyt, R. E. (1954) *J. Hyg., Camb.*, **52**, 100.
Felix, A.—(1938) *Ibid.*, **38**, 750.—(1952) *Ibid.*, **50**, 515.
Idem and Bhatnagar, S. S.—(1935) *Brit. J. exp. Path.*, **16**, 422.
Idem and Olitski, L.—(1926) *J. Immunol.*, **11**, 31.
Idem and Petrie, G. F.—(1938) *J. Hyg., Cqmb.*, **38**, 673.
Idem and Pitt, R. M.—(1934) *Lancet*, **2**, 186.—(1951) *J. Hyg., Camb.*, **49**, 92.
Henderson, S. W.—(1939) *Brit. J. exp. Path.*, **20**, 11.
Kauffmann, F.—(1951) *Enterobacteriaceae*. Copenhagen (Einar Munksgaard).
Kennedy, E. R. and Stuart, C. A.—(1950) *Proc. Soc. exp. Biol., N.Y.*, **73**, 538.
Landy, M.—(1954) *Amer. J. Hyg.*, **60**, 52.
Muschel, L. H. and Treffers, H. P.—(1956) *J. Immunol.*, **76**, 11.
Nagington, J.—(1956) *Brit. J. exp. Path.*, **37**, 385
Scholtens, R. Th.—(1937) *Zbl. Bakt.* (*Abt.* 1, *Orig.*), **139**, 467.
Topley, W. W. C. and Ayrton, J.—(1924) *J. Hyg., Camb.*, **23**, 198.
Webster, M. E., Sagin, J. F., Anderson, P. R., Breese, S. S., Freeman, M. E. and Landy, M.—(1954) *J. Immunol.*, **73**, 16.
Wheeler, K. M. and Stuart, C. A.—(1946) *J. Bact.*, **51**, 317.

MOUSE PROTECTION AND ENHANCEMENT OF PHAGOCYTOSIS BY ANTISERA TO *STREPTOCOCCUS PYOGENES*

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A strain of *Streptococcus pyogenes* may be tested for virulence by its capacity to kill mice when injected in small numbers, to resist phagocytosis by normal human leucocytes, and to grow freely in fresh normal human blood. Strains regarded as virulent by these tests have been found to contain the M antigen (Hirst and Lancefield, 1939), although the possession of an M antigen does not necessarily confer mouse virulence. Todd and Lancefield (1928) considered that immunity to streptococcal infections was M-type specific, since antisera containing M antibody protected mice against lethal doses of homologous types of streptococci. Such sera also enhance the phagocytic activity of human leucocytes and prevent free growth in normal human blood (Rothbard, 1945). Growth in rabbit or mouse blood is, however, not inhibited by M antisera. This species difference seemed to require investigation.

Evans (1941 ; 1943 ; 1946) made a great number of tests of the protective value of various antisera for mice and recognised several " immunogenic groups ", each comprising several different serological types, within which some cross-protection could be demonstrated. Although the general opinion has favoured Lancefield's view that protection is truly type-specific, no explicit test of Evans' results has been reported.

In planning a comparison of phagocytosis and bactericidal tests in human and mouse blood, it was decided to select a set of strains, some of which should, on Evans' hypothesis, show cross-protection.

EXPERIMENTAL

Strains.—The following strains and sera were used :

Type	Strain	Approximate LD_{50} (Number mouse passages in brackets)	Serum No.
6	S.43 (NCTC8302)	270 (3)	R/653 & R/651
18	J.17C (NCTC8320)	200 (33)	R/584
23	T.23 (NCTC8301)	10 (2)	R/604
1	S.F.130	127,000 (8)	R/595

Each strain was grown from freeze-dried cultures in 5 per cent horse blood broth ; these were injected intraperitoneally into mice. Twelve hours later the spleens were removed and the streptococci isolated by inoculating into blood broth. Estimates of the viable count of the cultures in each test were made by spotting 0·02 ml. of serial tenfold dilutions of culture (in 5 per cent broth saline) on blood agar plates containing $1/10^6$ crystal violet, each colony counted being one streptococcal unit.

All the strains gave marked precipitation reactions after 2 hr. at 37° when hydrochloric acid extracts were set up with fully-absorbed type-specific serum in capillary tubes (Swift, Wilson and Lancefield, 1943).

On nutrient agar enriched with 0·5 per cent glucose and 5 per cent horse serum all the strains except type 1 produced mucoid colonies.

Human blood was obtained from healthy human volunteers shown by previous tests to have no antibodies to the streptococcal types used. Ten ml. of blood was removed by venepuncture and added to 0·4 ml. of a 100 mg./100 ml. solution of heparin in isotonic saline.

Mouse blood was obtained by heart puncture from mice killed by coal gas. The blood was removed with a silicone-coated pipette under sterile conditions and added to 0·04 ml. of a 1000 mg./100 ml. solution of heparin in isotonic saline. Blood from 3 or 4 mice had to be mixed to obtain enough for the phagocytic counts.

Streptococcal antisera.—The sera used were those prepared in this laboratory by Lancefield's method (Lancefield, 1938), using a whole streptococcal cell vaccine, for the routine type identification of streptococci. Merthiolate (1/5000) was added for preservation and the sera were stored at 4°. Sera R/653 and R/651 were made in a similar way except that rabbit no. 651 received some doses of the whole killed vaccine made by disrupting the streptococci in a Michle disintegrator. In most of the tests the sera were used without any absorption; when the unabsorbed type 6 serum (R/651) was found to give cross-protection with the type 18 strain the tests were repeated with absorbed serum. The streptococcal types usually used as absorbing suspensions for producing specific anti-M sera are types 6 and 44. Antisera to types 1, 23 and 18 were therefore absorbed with a suspension of killed type 6. The sera then gave good specific precipitation reactions with acid extracts of the homologous streptococcal types used in the tests. Absorption of the type 6 antisera with a type 44 suspension, however, did not completely remove the cross reactions with type 18 extract but these were removed by further absorption with a suspension of type 36.

Thus each serum used was shown to contain anti-M substance and each strain used in the tests produced M-substance.

Mouse protection tests

Mouse protection tests were carried out with batches of 100 mice weighing 14–25 g. No modification of dosage of serum or culture was made for mice of different weights. The mice were selected at random and arranged in groups of five. Each group received one of the antisera as shown in Tables I and II, one group receiving no serum. The sera were injected subcutaneously into the back, in 0·5 ml. doses. Twenty-four hours later all the mice were given 0·5 ml. of an overnight broth culture of the streptococcus (diluted in 5 per cent broth saline) intraperitoneally. The ranges of the diluted cultures were from 10^{-2} to 10^{-5} or 10^{-4} to 10^{-7} depending on the virulence of the strain. All mice were observed for 10 days. Post mortem cultures of spleen and heart blood were taken from all animals dying after 2 days, or, in the second tests (Table II) from all the mice.

Phagocytosis tests

Phagocytosis mixtures were made in 2 × ½ in. (5 × 1·5 cm.) test tubes as follows:

Streptococcal antiserum or normal rabbit serum	0·02 ml.
Blood broth culture (18 hr.) of the streptococcus	0·02 ml.
Fresh mouse or human blood	0·3 ml.

The serum and the cultures were shaken for 1 min. and then the blood added. For each strain a control tube containing normal rabbit serum and four tubes containing antiserum against types 1, 6, 18 and 23 respectively were set up. All five tubes were placed in a Kahn shaker and agitated for 5 min. every 15 min. while being incubated at 37°. Duplicate blood films were made from each tube after 30 min. and after 2 hr., and stained with Leishman's stain. A total of 200 polymorph leucocytes was counted for each tube, and the proportion containing streptococci was recorded. Total white cell counts were made, using methyl-violet-acetic-acid diluting fluid, in a Neubauer counting chamber.

Blood-bactericidal tests

The bactericidal effect of human and mouse blood was estimated from mixtures similar to those employed for the phagocytosis tests, the culture being diluted 10^{-3} and 10^{-5}. The two

dilutions of culture were added to each of four tubes containing antistreptococcal sera of types 1, 6, 18 and 23 respectively with a control tube containing normal rabbit serum. These were shaken for 5 min. in every 15 min. on a Kahn shaker for 18 hr. at 37°. After 3 and 18 hr. duplicate loopfuls were plated on to blood agar containing $1/10^6$ crystal violet and incubated for 18 hr. at 37°. (Maxted, 1956.)

Capacity of human and mouse polymorphs to kill ingested streptococci

A mouse blood mixture was made as in the phagocytosis tests and incubated with shaking for 2 hr. at 37°. A drop of the preparation was then placed on an agar block under a microscope fitted with a wide angle $\frac{1}{6}$ in. phase contrast objective. A polymorph leucocyte which had ingested streptococci was drawn well away from the drop with the needle fitted to a Singer micromanipulator. The portion of the agar block holding the leucocyte was cut away and placed face downwards on a blood agar plate containing crystal violet, which was incubated at 37° for 18 hr. The whole procedure was repeated with human leucocytes. On both occasions the type 18 strain was used. I must thank Dr. J. E. Evans of King's College Hospital Medical School for performing the micromanipulations.

RESULTS

Mouse Protection Tests

With all four strains a high degree of protection of the mice was afforded by the homologous antiserum (Table I) and in these tests none of the heterologous

TABLE I.—*Mouse Protection Tests*

Streptococcal type injected and number of organisms in 10^{-4} inoculum.	Unabsorbed antiserum, type	Number of mice (of 5 inoculated) dead within 10 days of inoculation with culture diluted.						Overall mortality per cent
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
T.23 (14,000)	None	—	—	5	5	5	4	95
	23	—	—	3	0	0	0	15*
	18	—	—	5	5	4	3	85
	6	—	—	5	5	5	5	100
	1	—	—	5	5	5	4	95
T.6 (27,000)	None	—	—	4	5	3	0	60
	6	—	—	2	0	0	0	10*
	23	—	—	5	4	4	2	75
	18	—	—	5	4	5	3	85
	1	—	—	4	5	3	1	65
T.18 (11,000)	None	5	5	2	4	—	—	80
	18	3	0	1	1	—	—	25*
	23	5	5	2	3	—	—	75
	6 (R/653)	5	3	4	3	—	—	75
	1	5	4	2	2	—	—	65
T.1 (12,700)	None	—	1	2	2	0	—	25
	1	—	0	0	0	0	—	0
	23	—	5	3	0	0	—	40
	18	—	3	4	0	1	—	40
	6	—	2	1	1	0	—	20

— = test not done.
* = percentage differing from control by more than 3 times the standard error of the difference

sera gave any substantial protection. With type 18 however the type 6 serum R/653 and the type 1 serum R/595 seemed to delay the death of the animals. A second test was therefore carried out (upper half of Table II).

For this test a new type 6 serum R651 had to be used; it gave significant protection against infection with the type 18 streptococci. The serum was then

absorbed with type 44 strain. Although this absorbed serum gave no more than a trace of precipitate with extracts of most types, it gave a moderately strong reaction with extracts of type 18 and type 36. It was therefore re-absorbed with type 36, which removed all trace of heterologous precipitation. When the fully absorbed serum was used in a mouse protection test it no longer protected against type 18 infection (Table II). Thus a relationship was shown between types 6

TABLE II.—*Mouse Protection Tests*

Streptococcal type injected and number of organisms in 10^{-4} inoculum	Antiserum type	Number of mice (of 5 inoculated) dead within 10 days of inoculation with culture				Overall mortality per cent
		10^{2}	10^{-3}	10^{-4}	10^{-5}	
T.18 (13,000)	Normal serum	5	3	3	5	80
	18	3	4	2	2	55
	6 (R/651)	3	2	1	1	35*
	1	5	4	3	2	70
	23	5	5	3	3	80
T.18 (8,400)	Normal serum	4	5	5	—	93
	18	3	3	2	—	53†
	6 (R/651)	4	4	3	—	73
	6(R/651) Absorbed	5	5	3	—	87

— = test not done.
* = percentage differing from control by more than 3 times the standard error of the difference.
† = " " " " " " " " 2 " " " " " " " "

and 18 by mouse protection and precipitation tests using type 6 serum R/651.

These results suggest that the heterologous reaction observed in the precipitin test might be due to an antibody, other than M, which could protect mice and enhance phagocytosis.

Phagocytosis Tests

Phagocytosis was always considerably enhanced by homologous serum within 30 min. of the beginning of incubation (Table III). Only one of the tests showed significant enhancement of phagocytosis by heterologous serum, namely that with type 18 streptococci and type 6 (R/651) serum in mouse blood. This was the serum which protected mice against type 18 infection. When this phagocytosis test was repeated, using the type 6 R/651 serum absorbed with streptococci of types 44 and 36 to remove all precipitation cross-reactions, no enhancement of phagocytosis of type 18 streptococci was observed. In a repetition with the unabsorbed R/651 serum the enhancement was less than in the first test but still greater than with the control.

The results of the phagocytosis tests in mouse blood thus conformed with those of the protection tests: antisera which protected mice against infection with homologous or heterologous types also enhanced phagocytosis. On the other hand, no significant enhancement of phagocytosis by heterologous antiserum could be demonstrated in the human phagocytosis tests, although homologous sera always gave good enhancement.

Blood-bactericidal Tests

All four streptococcal strains resisted the bactericidal effect of normal human blood in the presence of normal rabbit serum or of a heterologous serum. With homologous serum the growth of all the strains was inhibited at 3 hr. and 18 hr.

TABLE III.—*Phagocytosis Tests*

Infecting streptococcus :	Percentage polymorphs containing streptococci											
	Type 23		Type 6		Type 1		Type 18		Type 18		Type 18	
Blood :	Mouse	Human	Mouse	Human	Mouse	Human	Mouse	Human	Mouse	Human	Mouse	Human
Streptococcal : polymorph ratio	4 : 1	5 : 1	17 : 1	1·5 : 1	15 : 1	3 : 1	5 : 1	1 : 1	3·7 : 1	1·7 : 1	1·9 : 1	1·2 : 1
Time of sample (hr.)	½	½	½	½	½	½	½	½	½	½	½	½
Control	4·5	33	0·6	0·5	10	6·8	7·5	9·8	2·3	3·5	3	11
Mixtures + antiserum :												
1	6·0	37·5	0·5	0·5	51	46·3	3·0	3·4*	3·7	8·0	—	—
18	4·5	15·5*	0·7	1·0	6·7	9·5	33·4	44	28·7	76·5	50	30
6 (R/653)	4·5	22*	28	51·5	8·3	9·3	5	8·3	—	—	—	—
23	24·5	67·5	1·6	3·0	5·0	10·3	4·5	5·4*	3	5·5	—	—
6 (R/651)	—	—	—	—	—	—	—	—	11·3†	6·5	7	9
6 (R/651) (absorbed T44 and T36)	—	—	—	—	—	—	—	—	—	—	2	10

The significance levels of counts from heterologous serum mixtures are indicated as follows :
— = test not done.
* = less than the control by more than 2 S.E.
† = more than the control by more than 2 S.E.

Only 30 min. percentage counts are shown ; these correspond with the 2 hr. values except with type 23 streptococcus in human blood. In this test the mixture containing type 1 antiserum showed a significantly high percentage at 2 hr.

With mouse blood none of the strains was inhibited even in the presence of homologous antiserum. Growth curves of type 18 streptococci in mouse and human blood with and without homologous antiserum are shown in Fig. 1 and 2.

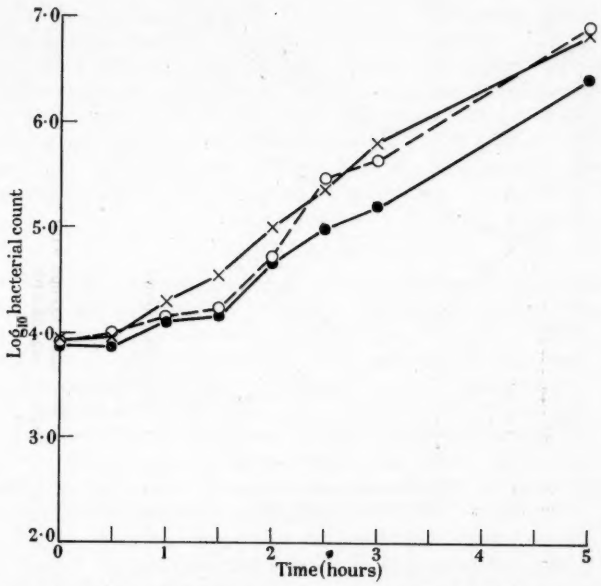

FIG. 1.—Growth curves of *Streptococcus pyogenes* type 18 in:— ×———× broth, ○- - - - -○ mouse blood + heterologous serum, ●———● mouse blood + homologous serum.

Viable counts were made from ten-fold dilutions of the mixtures used in the phagocytosis test on blood agar containing crystal violet. The growth rate of the type 18 strain in mouse blood was only slightly reduced (probably due to the effect of agglutination) by homologous serum. With human blood, streptococci were always killed in the presence of homologous antiserum. It was thought that the poor bactericidal capacity of mouse blood might have been due to the relatively low total polymorph leucocyte count of normal mice. Fresh heparinised mouse blood was therefore spun at 1500 r.p.m. for 10 min. and 0·3 ml. of the buffy coat was removed and mixed with 0·02 ml. of streptococcal culture (type 18) and 0·02 ml. of homologous antiserum. The same mixture with a heterologous serum was made as a control. Concentrating the mouse blood in this way gave a total polymorph count of 3000/cu. mm. which is similar to that in human blood, but this increase in the number of polymorphs did not reduce the growth rate of the streptococci (Fig. 3). It was not practicable to make parallel phagocytosis and viable counts on the same mixtures because of the vastly different leucocyte : streptococcus ratio : in the growth-curve experiments the initial streptococcal concentration was 10,000 units/ml., the organism : polymorph ratio being 1 : 400 ; in the phagocytosis experiments the initial strepto-

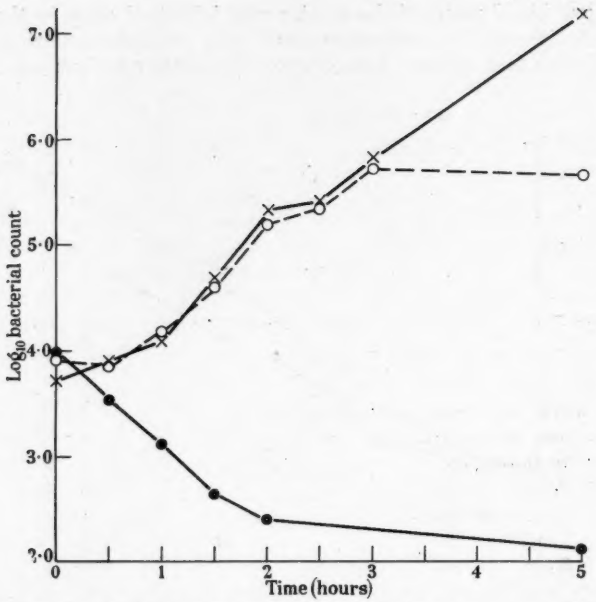

FIG. 2.—Growth curves of *Streptococcus pyogenes* type 18 in:— ×———× blood broth, ○- - - - -○ human blood + normal rabbit serum, ●———● human blood + T18 unabsorbed rabbit serum.

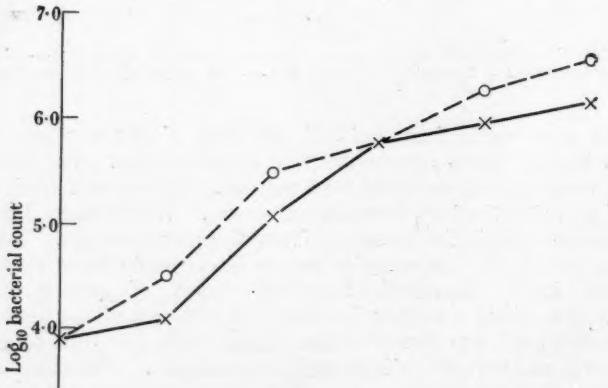

FIG 3.—Growth curves of *Streptococcus pyogenes* type 18 in concentrated mouse white cell suspension:— ×———× + homologous serum, ○- - - - -○ + heterologous serum.

coccal concentration was 20 × 10^6 units/ml., with an organism : polymorph ratio of up to 20 : 1.

The Fate of Streptococci Ingested by Polymorphs

Since the capacity of mouse blood to kill streptococci was not increased by raising the total polymorph count, it was thought that mouse polymorphs might be less capable of killing ingested streptococci than human. Mouse polymorphs which had ingested type 18 streptococcal chains were therefore separated with a micromanipulator from extracellular streptococci and incubated on blood agar containing crystal violet ; 5 out of 7 such cells gave colonies of type 18 streptococci. When human polymorphs were similarly manipulated only 2 out of 8 cells gave colonies. Although the difference is not significant this suggests that human polymorphs may kill ingested streptococci more easily than mouse polymorphs.

DISCUSSION

Previous work on mouse protection with heterologous sera against types of *Str. pyogenes* has given variable results. Lancefield showed that mice were not protected by heterologous antisera to any great extent. Mudd, Czarnetsky, Lackman and Pettit (1938) performed tests with small numbers of mice and obtained cross-reactions which they attributed to " C " substance. Evans seems to be the only worker who has used a large number of strains, but many of her tests were made with small numbers of mice, and the type identification was based solely on slide agglutination. Moreover, she could not demonstrate protection with heterologous sera with all the strains in her " immunogenic " group containing *Str. pyogenes* types 6 and 18.

The experience recorded here may explain these discrepancies, in that the capacity to protect against infection with a heterologous type was observed with only one of two sera prepared against a single strain of streptococcus. The cross-protection with the one serum was confirmed by its enhancement of phagocytosis in mouse blood, and it was noteworthy that the serum gave a moderately strong precipitin reaction with an acid extract of the heterologous strain in question. The second serum, which differed only in that it was derived from a different rabbit which had had some doses of the vaccine strain prepared by disruption of streptococci, showed no significant protection or enhancement of phagocytosis but it did delay death. It may be that heterologous protection only appears when certain streptococcal antibodies, other than anti-M substance, are present in sufficient concentration.

Mouse blood had no bactericidal effect on *Str. pyogenes* in the presence of rabbit antiserum against the same type, even though this serum was able to protect mice against the infection. Nevertheless phagocytosis was enhanced by homologous serum and in one case by heterologous serum. Given a high enough streptococcus : polymorph ratio, mouse polymorph leucocytes (in the presence of homologous serum) ingested the organisms but appeared less capable of killing them than human leucocytes. This lack of bactericidal effect might be due to the streptococci growing extracellularly faster than they were being ingested, but further work would be needed to elucidate this. Presumably the killing of streptococci injected into serum-protected mice is performed by phagocytes in the liver and spleen more effectively than in the blood.

SUMMARY

Antisera against four strains of different types of *Str. pyogenes* were tested for protection of mice infected with heterologous types. In one test such cross-protection was observed but with only one of two specimens of antiserum prepared in different rabbits against the same vaccine strain.

Phagocytosis tests with mouse blood conformed with the mouse protection tests, but, unlike human blood, mouse blood was not bactericidal even with the addition of streptococcal antiserum. Some evidence is presented that mouse polymorphs are less capable than human polymorphs of killing engulfed streptococci.

I am indebted to Dr. P. Armitage of the Medical Research Council's Statistical Research Unit for his help with the statistics, to Mr. D. P. Woodroof for his technical assistance, and to Miss J. Stubbs for the preparation of the sera.

REFERENCES

Evans, Alice C.—(1941) *J. Immunol.*, **42**, 15.—(1943) *Ibid.*, **46**, 399.—(1946) *Ibid.*, **52**, 1.

Hirst, G. K. and Lancefield, Rebecca C.—(1939) *J. exp. Med.*, **69**, 425.

Lancefield, Rebecca C.—(1938) *Proc. Soc. exp. Biol., N.Y.*, **38**, 474.

Maxted, W. R.—(1956) *Brit. J. exp. Path.*, **37**, 415.

Mudd, S., Czarnetsky, E. J., Lackman, D. and Pettit, H.—(1938) *J. Immunol.*, **34**, 117.

Rothbard, S.—(1945) *J. exp. Med.*, **82**, 107.

Swift, H., Wilson, Armine T. and Lancefield, Rebecca C.—(1943) *Ibid.*, **78**, 127.

Todd, E. W. and Lancefield, Rebecca C.—(1928) *Ibid.*, **48**, 751.

THE INDIRECT BACTERICIDAL TEST AS A MEANS OF IDENTIFYING ANTIBODY TO THE M ANTIGEN OF *STREPTOCOCCUS PYOGENES*

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THE accepted method for the identification of serotypes of *Streptococcus pyogenes* (Lancefield Group A streptococci) is based on a precipitin reaction with antisera against the type-specific M antigen. The criteria for an anti-M serum are that it should give precipitation with the acid-extracted antigen of the vaccine strain, and of other strains of the same "type", and should not react with such extracts after they have been treated with trypsin; and that it should confer passive protection on mice when given twenty-four hours before a challenge dose of the organism.

Many workers who have had experience of the preparation of type-specific precipitating sera have come across non-specific trypsin-sensitive antigens which might be confused with M substance. It is also a common experience to find that certain type sera are always difficult to make; such types as 8, 11, 13, 22, 25, and 27 have never given satisfactory precipitating sera (Williams and Maxted, 1953). Highly mouse-passaged variants of these types sometimes produce antisera which, after absorption, precipitate well with extracts of the vaccine strain but not with extracts of the same strain before mouse passage.

When the nature of the antibody is in doubt, the ability to confer passive protection on mice is the most reliable method of establishing whether it is an M antibody. However, because of the low virulence of so many of the vaccine strains it is often impossible to carry out the mouse protection test. This is often so even though the strains have been freshly isolated from cases of acute streptococcal infection in man.

The bactericidal test has been used to show the development of protective antibody in the serum of patients convalescent from streptococcal infection. The direct bactericidal test uses the patient's whole blood (Todd, 1927) and the indirect test makes use of the blood from normal children, to which a small amount of patient's serum is added (Rothbard, 1945). The M antibodies thus introduced confer bactericidal powers upon the blood against streptococci of the same M type. It seemed possible that such a test might well be a substitute for mouse protection in the testing of anti-M rabbit sera. The main points of interest were to identify M antibody reacting with avirulent strains, to use the same technique to identify M antibody made from possible new types of streptococci, and to

study the reactions of strains having a trypsin-labile antigen which gave only moderate and possibly non-specific precipitation with type antisera.

MATERIALS AND METHODS

Strains

These were of three classes.

Class I.—Strains used to make some of the accepted anti-M sera which have always been easy to prepare. These strains have been subdivided into two classes, I*a* virulent for mice and I*b* non-virulent.

Class I*a*	Type	1	R154	Type	30	D24/46
		17	J17E/19		36	C119
		19	J17D/50		39	C95
Class I*b*	Type	5	T5B	Type	9	41445/10

Class II.—Recognised type strains, the M antisera of which have nearly always proved difficult to prepare, and about which there has often been doubt as to the true nature of the reaction obtained.

Type	2	T2/44/Rb5	Type	8	SF4matt
	4	R54/2692		22	45128

All these were avirulent for mice.

Class III.—Provisional new types, the antisera to which, though reacting with extracts of the homologous strains, also reacted with trypsin-labile antigens extracted from cells of other types.

Provisional types : " Lily ", " Angas " and " Corby ".

Antisera

The sera were prepared in this laboratory by the usual method : rabbits were injected with heat-killed suspensions of washed cells on three successive days in each week.

Blood

Fresh normal human adult blood was used. The donors were selected because their blood allowed the multiplication of the particular strains. All blood was heparinised (8 units/ml.). Blood from normal Rhesus monkeys was used in some experiments.

Bactericidal test

Corked sterile tubes 2 in. × ½ in. (5·1 × 1·3 cm.) were set up in Wassermann racks and 0·3 ml. of heparinised blood was run into each followed by 0·02 ml. of rabbit anti-M serum which was added to each tube with a dropping pipette. Normal rabbit serum or saline was added to a control set. Overnight cultures of the streptococcus were diluted in 10 per cent broth saline and 0·02 ml. of diluted culture was added to the appropriate mixture. The usual test range dilution of culture was 10^{-5}, 10^{-4}, 10^{-3}. The number of viable streptococcal units in 0·02 ml. of highest dilution averaged between 50 to 100. The mixtures were put into a Kahn shaker set to shake for 3 min. every 15 min. with a time clock. The wide rounded base of the tubes allowed adequate mixing.

The mixtures were explanted at 3 hr. and 20 hr. A large standard loop was filled with blood mixture and spread evenly over ¼th sections of duplicate blood agar plates which were incubated overnight and the growth recorded over the range — to ++++.

RESULTS

Glossy variants of streptococci failed to grow in normal blood while the matt M-containing variants usually grew readily. It was a fair assumption that strains

capable of growing contained M antigen, and in all cases where well-established type strains were used and grew freely, this was so.

The use of rabbit serum in human blood mixture

Normal human blood was tested against class I*a* M type strains ; blood that allowed all the type strains being investigated to grow was selected. Each strain was set up against mixtures containing normal rabbit serum, the homologous antiserum and at least one heterologous antiserum.

Explants were made at 3 hr. and 20 hr. The latter period of incubation provided a rather severe test, but it was found that with good anti-M sera the blood was usually able to kill the organisms completely or to restrict their growth to a minimum. The results using three known types, 17, 18, and 30, are shown in Table I.

Such mixtures of serum and normal human blood were highly bactericidal and the results were reproducible. Other good anti-M rabbit sera (Types 24, 36, and 39) were used in the same way and proved equally satisfactory.

Blood from other species

The necessary search for a suitable normal human blood is tiresome. Rabbit blood, even from rabbits that provided excellent M antiserum, had proved to have no bactericidal action whatever, even for the streptococcus with which they had been inoculated. Monkey blood was therefore tested.

Heparinised blood obtained by heart puncture of the monkeys was supplied by the Virus Reference Laboratory, and was used in exactly the same way as in the successful tests with human blood. The results with the first two animals are shown in Table II ; clearly the blood from these was quite suitable. Of the next two animals used one yielded satisfactory blood while that of the other behaved in the same way as rabbit blood. No increase in killing power was obtained by adding anti-M serum. However it seems possible to obtain a satisfactory animal.

*Avirulent M strains and their antisera (Class I*b)

The strains used in the experiment reported in Tables I and II were either mouse-virulent or could easily be made so and the sera were all well established anti-M sera. These strains and sera could therefore have been tested just as well by mouse protection tests. This was not the case with strains of Class I*b* represented here by type 5 and type 9 (Table III).

Acid extracts of type 5 reacted well with the antiserum. The antiserum made against type 9 gave moderate precipitation with the homologous extract but did not react with all type 9 strains encountered. Both strains were avirulent for mice but were able to multiply when inoculated into suitable human blood. The indirect bactericidal test showed that antisera made from both strains contained a phagocytosis-enhancing antibody but that the type 9 antiserum was rather weak in this respect.

The usefulness of the test using non-virulent strains has been confirmed with other well established types, *e.g.*, type 1, type 6 and type 29.

Avirulent strains producing doubtful M antisera (Class II)

The strains and sera in this series were of types 2, 4, 8, and 22 (Table IV). The type 2 serum gave good precipitation, but not as many freshly isolated type 2 strains identified by agglutination reacted with it as were expected.

The type 2 serum R434, tested against two different type 2 strains, had a bactericidal effect and this indication that it contained M antibody confirmed earlier work with this serum (Maxted 1953). Type 4 was of particular interest since the absorbed serum precipitated moderately well with most strains identified as type 4 by agglutination. Extracts of these strains however often reacted with sera of related types such as 29 and 46, which would not be expected with a true M antigen. Not many type 4 strains grew well in human blood, but when strains that grew freely were used in an indirect bactericidal test with the antiserum, there was no evidence of enhanced phagocytosis, which suggested that the precipitation reaction was not due to type 4 M antibody. This has been confirmed by other work done in this department (Hambly, in preparation).

The type 22 serum reacted with extracts of the vaccine strain only but the bactericidal test failed to show any enhanced killing power. Type 8 antiserum also failed to promote bactericidal action but this was expected since it gave poor and only non-specific precipitation with acid extracts of the streptococci. None of the strains in this series was virulent enough to be used as a challenging strain in protection tests in mice.

Lancefield and Perlmann (1952) have shown that the " type 28 " R antigen is quite unlike the typical M antigen : it is resistant to trypsin digestion and its antibody fails to give passive protection. Many strains have the R antigen but in only two strains has there been a suggestion that the more usual M-like antigen was also present. These were two strains which Lancefield and Perlmann had rendered highly mouse-virulent after numerous mouse passages. Antisera against the passaged strains protected mice against challenge with them.

In previous work here, attempts had been made to demonstrate a typical M antigen in stock strains of type 28 giving strong R-antigen reactions ; many sera were made, all of which contained much R antibody and some of which also gave precipitation with trypsin-sensitive protein fractions extracted from the streptococcus. However, bactericidal tests with these strains and their antisera showed no enhancement and so failed to confirm that the antibodies were the result of stimulation by M protein. Parallel tests could not be done in mice because the strains failed to increase in virulence even after numerous mouse passages.

New types giving reproducible and characteristic T agglutination (Class III)

Three strains known as " Lily ", " Angas " and " Corby " were recognised by their characteristic agglutination patterns in an epidemiological study in a children's home (Holmes and Williams, in preparation). They failed to precipitate with any type antisera. Attempts were therefore made to prepare specific sera for these three strains. One antiserum against " Lily " precipitated well with the homologous strain but also with a large variety of strains which, according to the agglutination pattern, were unrelated. The second serum, against " Angas ", was specific in its precipitation but no strains have so far been found outside the community under investigation. The serum against the third strain, " Corby ",

TABLE I.—*Inhibition of Growth of Streptococci in Human Blood to which Anti-M Rabbit Serum had been Added*

Addition to blood.	Culture dilution. Type 17. 10^{-5}.	Type 17. 10^{-4}.	Type 17. 10^{-3}.	Type 19. 10^{-5}.	Type 19. 10^{-4}.	Type 19. 10^{-3}.	Type 30. 10^{-5}.	Type 30. 10^{-4}.	Type 30. 10^{-3}
Saline . .	+/++++	+++/++++	++++/++++	+/+++	+++/++++	+++/+++	±/++++	++/++++	+++/++++
Type 17 antiserum	−/−	−/++	±/+++	−/++++	++/++++	+++/+++	±/++++	+/++++	+++/++++
,, 19 ,,	+/++++	++/++++	+++/++++	−/−	±/−	−/−	±/++++	+/++++	++/++++
,, 30 ,,	±/++++	++/++++	+++/++++	+/++++	++/++++	+++/++++	−/−	−/−	±/−

In this and the following Table : − = no growth ; ± . . . ++++ increasing amounts of growth. The symbol before the stroke refers to the sample taken after 3 hr. incubation, that after the stroke to the sample taken after 20 hr.

TABLE II.—*Inhibition of Growth of Streptococci in Monkey Blood to which Anti-M Rabbit Serum had been Added*

Addition to blood.	Culture dilution. Type 17. 10^{-5}.	Type 17. 10^{-3}.	Type 19. 10^{-5}.	Type 19. 10^{-3}	Type 30. 10^{-5}.	Type 30. 10^{-3}.
Monkey No. 11						
Saline	+/++++	+++/++++	±/++++	+++/++++	±/++++	+++/++++
Type 17 antiserum .	−/−	±/±	+/++++	+++/++++	+/++++	+++/++++
,, 19 ,, .	+/++++	+++/+++	±/−	±/++	+/++++	+++/++++
,, 30 ,, .	+/++++	+++/++++	+/++++	+++/++++	−/−	±/±
Monkey No. 12						
Saline	+/++++	+++/++++	±/++++	+++/++++	++/++++	+++/++++
Type 17 antiserum .	−/±	±/±	±/++++	+++/++++	N.D.	N.D.
,, 19 ,, .	+/++++	+++/++++	−/−	±/−	N.D.	N.D.
,, 30 ,, .	++/++++	++++/++++	+/++++	+++/++++	−/−	±/−

N.D. = not done.

TABLE III.—*Bactericidal Test of Non-virulent Strains with their Type Sera Containing Known M Antibody*

Addition to blood.	Culture dilution. Type 5. 10^{-5}.	Type 5. 10^{-4}.	Type 9. 10^{-5}.	Type 9. 10^{-4}.
Normal rabbit serum	+/++++	+++/++++	+/++++	++/++++
Type 5 antiserum	−/+	−/+	N.D.	N.D.
„ 9 „	N.D.	N.D.	+/−	+/++
Heterologous type serum	+/++++	+++/++++	±/+++	+++/++++

TABLE IV.—*Bactericidal Test of Non-virulent Strains with their Antisera, which Contain Doubtful M Antibodies*

Addition to blood.	Culture dilution. Type 2. 10^{-5}.	Type 2. 10^{-3}.	Type 4. 10^{-5}.	Type 4. 10^{-3}.	Type 8. 10^{-5}.	Type 8. 10^{-3}.	Type 22. 10^{-5}.	Type 22. 10^{-3}.
Normal rabbit serum	±/++++	+++/++++	+/++++	++/++++	+/+	++/++++	±/++++	++/++++
Homologous type serum	−/±	−/±	±/++++	++/++++	+/+	++/++++	−/++++	+/++++
Heterologous type serum	±/++++	++/++++	±/++++	++/++++	++/++++	+++/++++	+/++++	+++/++++

TABLE V.—*Serological Reactions and Bactericidal Test of Provisional New Types with their Antisera*

Addition to blood.	Lily. 10^{-5}.	Lily. 10^{-3}.	Angas. 10^{-5}.	Angas. 10^{-3}.	Corby. 10^{-5}.	Corby. 10^{-3}.
Normal rabbit serum	+/+	++/+++	+/+	++/+++	++/++++	+++/++++
Homologous serum	+/−	+++/+++	−/−	±/−	−/−	−/−
Heterologous serum	++/++	+++/+++	+/++	+++/+++	+/+++	+++/++++
Range of precipitin reactions of serum	2/3/9/11/44/Lily		Angas		Corby	
Typical agglutination pattern of strain	3/13/B3264/12/27/Lily		11/27/44/Angas		5/27/44/Corby	

In the bactericidal test unabsorbed sera were used. For the agglutination and precipitation tests the sera were absorbed to remove non-type specific antibody.

precipitated well with the homologous type but was also prone to show cross reactions with some heterologous types.

Bactericidal tests (Table V) suggest that " Angas " and " Corby " are in fact new types, but the serum of strain " Lily " was as unconvincing in bactericidal tests as in precipitation with acid extracts. It seems unlikely that precipitates obtained with this strain are indeed due to M antigen antibody reaction. The strains were all avirulent for mice.

DISCUSSION

The bactericidal test can be used as an alternative to protection tests with mice for detecting M antibody. Many strains that cannot be used in mouse protection tests are usable in the bactericidal test. Although not all strains that contain M antigen will grow freely in human blood apparently free from specific antibodies, it is generally easier to find a suitable strain and blood than to raise the virulence of a strain by a long series of passages through mice.

It is interesting to find that Rhesus monkey blood may be used for testing Group A organisms. This seems to be the only example of an animal other than man that is suitable, and it is the only animal in which natural carriage of Group A streptococci has been observed.

In view of the consistent results obtained with strains known to give good M antisera, the failure of some types (4, 8 and 22) to produce bactericidal antisera confirms our suspicions that the precipitation results obtained with these sera are not due to M antigen.

In our hands stock strains of type 28 have failed to show any trace of M antigen in spite of yielding precipitinogens which can, like true M antigen, be destroyed by proteinase digestion. However these tests do not rule out the possibility that a true M antigen may be present in these strains.

The bactericidal test enables a large number of strains to be tested against many sera and there is no doubt that by its use some provisional M-types may well be discarded and equally certain that some, whose antisera give bactericidal action, will repay the additional investigations these results may stimulate. It is evident that the possession of M antigen alone is not enough to enable a strain to multiply in all human blood apparently devoid of antibody, and experience gained by the extended use of the bactericidal test may focus attention on other streptococcal fractions which play a part in virulence.

It is the custom to pass streptococci through mice to promote their virulence and increase their M content, yet many of these strains contain ample amounts of M antigen in their original form but only stimulate antibody production after mouse passage. The mouse passage, which is quite foreign to the streptococcus, may promote the development of a false precipitating antigen. Bactericidal tests, by representing a more normal environment, may give a more reliable index of the pathogenic abilities of some strains of *Str. pyogenes*.

SUMMARY

The ability of antiserum prepared in rabbits to enhance the bactericidal power of normal human blood for streptococci of the homologous type runs closely parallel with its ability to protect mice against intraperitoneal challenge. Monkey blood has also proved suitable for this purpose.

By this method it is possible to test many strains which are avirulent for mice yet contain ample M antigen, and also many sera which give M-like precipitations with acid extracts of the streptococcus but which probably contain a non-specific antibody.

REFERENCES

LANCEFIELD, REBECCA C. AND PERLMANN, GERTRUDE E.—(1952) *J. exp. Med.*, **96**, 83.
MAXTED, W. R.—(1953) *J. Path. Bact.*, **65**, 345.
ROTHBARD, S.—(1945) *J. exp. Med.*, **82**, 93.
TODD, E. W.—(1927) *Brit. J. exp. Path.*, **8**, 1.
WILLIAMS, R. E. O. AND MAXTED, W. R.—(1953) *Atti del Congresso Internationale di Microbiologia Roma*, **1**, 46.

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